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Surveillance of potentially pathogenic amoebae in Essex County waters.

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SURVEILLANCE OF POTENTIALLY PATHOGENIC AMOEBAE IN ESSEX COUNTY WATERS

by

Maria Vrzoc

A thesis
submitted to the Faculty of Graduate Studies
through the Department of
Biology in Partial Fulfillment
of the Requirements for the Degree
of Master of Science at
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1984

ABSTRACT

Surveillance of Potentially Pathogenic
Amoebae in Essex County Waters

Free–living, freshwater and soil amoebae, of the genera *Acanthamoeba* and *Naegleria*, are the causative organisms of the conditions known as granulomatous amoebic encephalitis (GAE) and primary amoebic meningoencephalitis (PAM), respectively, in man. GAE is a condition characteristic of individual whose immune systems have been suppressed, either through illness or drug therapy. These infections are not always fatal. Persistent chronic infections as well as spontaneous remissions may occur. PAM infections are generally found in young, active persons with a recent history of exposure to water. The infections are almost always fatal.

Evidence exists that thermal pollution of water, either natural or man–made, and pollution from human sewage, result in conditions favorable for the proliferation of these pathogenic amoebae. As human populations increase in size, the demands on available freshwater, for power production, recreation and as a dumping ground for sewage, also increase. Conditions hazardous to the health of these populations may result. This study examined twelve recreational beaches in Essex County for the presence of these potentially pathogenic amoebae. The effects of thermal pollution from an electric power plant were also studied. A primary and secondary waste treatment plant in Essex County were monitored for their relative efficiencies in eliminating high temperature tolerant amoebae (HTTA), i.e., amoebae which could grow at 44.5° C, *Acanthamoeba* which could grow at 41° C, and *Naegleria*. Waste stabilization ponds, at two communities, were checked for a role as potential reservoirs for these amoebae. Finally, various biological, chemical and physical parameters were measured to determine whether they could be used as indicators of the presence of these three types of amoebae.

Of a total of 428 beach samples taken in 1982, 81 (18.93 %) contained HTTA, 66 (15.42 %) contained *Acanthamoeba* and 4 (0.93 %) contained *Naegleria*. In 1983, of 308 beach samples taken the results were: 92 (29.87 %), 66 (21.43 %) and 10 (3.23 %), respectively. No *Naegleria* isolate was found to be pathogenic in mice when injected intranasally. Mouse pathogenicity of HTTA and *Acanthamoeba* was not tested.

There was a general increase in the number of positive samples over the sampling period, for all three types of amoebae. These increases accompanied increases in water temperature. Similar results were obtained during both years of the study. A higher percentage of sediment samples were found to contain these amoebae than water samples taken at the same location. This suggests greater concentrations of these amoebae in sediments.

Both waste treatment plants and waste stabilization ponds contained HTTA, *Acanthamoeba* and *Naegleria*. The presence of these amoebae in effluents suggested that these locations may act as reservoirs and dispersal points for potentially pathogenic amoebae. The primary waste treatment plant was not as efficient at eliminating these amoebae as the secondary waste treatment plant.

The effect of thermal effluents on populations of HTTA and *Acanthamoeba* was minimal. Discharge waters from the J. C. Keith Power Plant were not able to raise water temperatures, to a high enough level or for a long enough period, to allow the growth of either pathogenic or non-pathogenic *Naegleria*.

Parameters measured were not able to predict the presence of potentially pathogenic amoebae, although they were somewhat useful in explaining the presence or absence of these amoebae in waters tested.

Although populations of pathogenic amoebae are not large enough to be a potential danger at present, cultural eutrophication may result in conditions favorable to their proliferation. It is recommended, therefore, that a permanent monitoring system be established, especially at beaches on Lake Erie.

ACKNOWLEDGEMENTS

I would like to express my gratitude to all those responsible for making this thesis possible. First, thanks are given to the members of my committee: my advisor, Dr. J. M. Winner, Dr. D. Cotter and Dr. K. Taylor for the time they spent reviewing this work. In addition, I would like to thank my fellow graduate students in Dr. Cotter's laboratory for allowing me to make their laboratory my second home and giving me the benefit of their support and advice. I would also like to acknowledge Mr. Diwakar Gupta from the Engineering Department for his assistance with my computer work. Finally, I would like to thank my parents for their financial and emotional support during this work.

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INTRODUCTION

Free–living amoebae of the genera *Acanthamoeba* and *Naegleria* may be pathogenic when injected into humans or test animals by routes other than the gastrointestinal tract. Culbertson *et al.* (1959) first reported a case in which fluid from a culture of monkey kidney cells, suspected of harboring an unknown virus, caused the death of test animals when injected intracerebrally. Autopsies revealed the presence of amoebae in brain lesions. Subsequent intranasal installation of a pure culture of the isolate resulted in fatal meningoencephalitis. The causative organism was found to be a free–living freshwater and soil amoeba of the genus *Acanthamoeba*. Subsequent fatalities from these organisms have been reported, either during the course of the illness or upon postmortem histological examination. Culberson (1971) reviewed the studies resulting from his initial findings. Fowler and Carter (1965) and Butt (1966) first recognized the occurrence of primary amoebic meningoencephalitis (PAM) in humans. Amoebae of the genus *Naegleria* are associated with this disease.

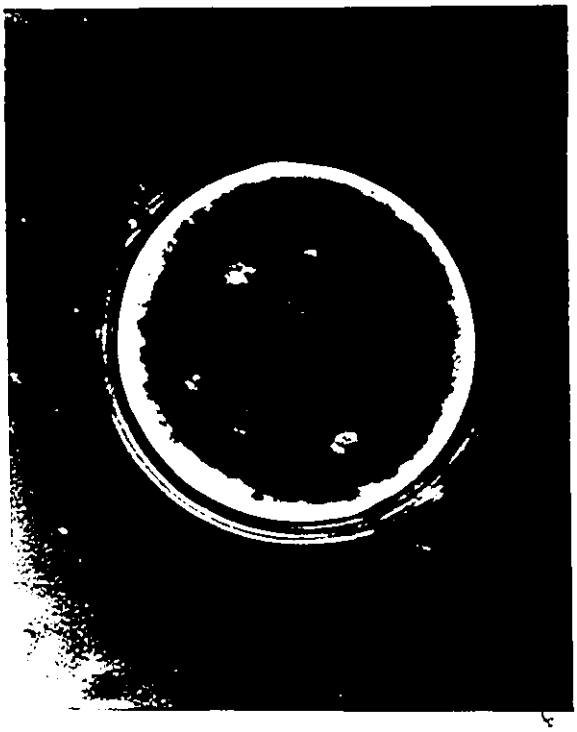
A. Life Cycle and Morphology

There are two stages in the life cycle of *Acanthamoeba*; (a) a vegetative trophozoite (Figure 1A), which generally feeds on bacteria, and (b) a cyst stage (Figure 1B), resistant to desiccation and other unfavorable environmental conditions. In addition to these two stages (Figures 2A, 2B), *Naegleria* possess a transient flagellate stage (Figure 2C).

The two genera are characterized by a nucleus containing a large and central nucleolus. Trophozoites have characteristic shapes during locomotion with *Naegleria* having smoother outlines and more rapid movement than *Acanthamoeba*. Trophozoites of *Acanthamoeba* are characterized by

Figure 1. Stages in the life cycle of *Acanthamoeba*. (A) trophozoite. (B) cyst. Both stages are shown at the same magnification (x1250) and were photographed using a PM-10AD Photomicrographic system on an Olympus Vanox microscope. The arrows, in both figures, indicate the single nucleus with a large, central nucleolus. Note the chromatin material on the periphery of the nuclear membrane.

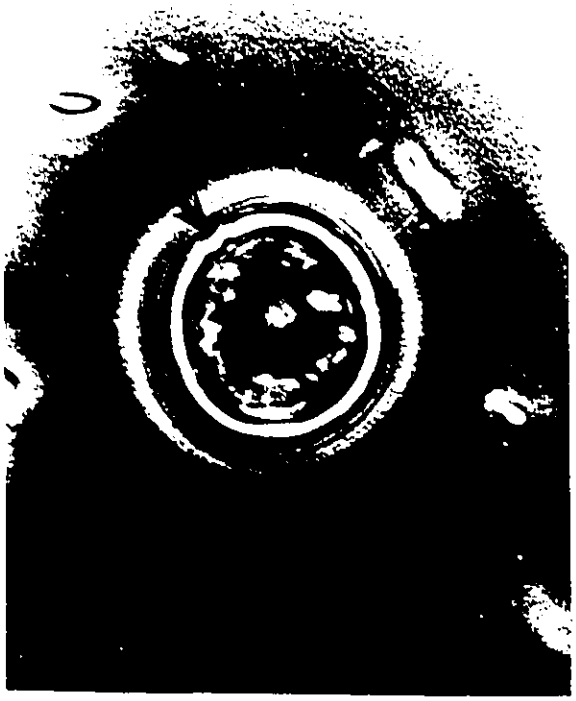
A



B

Figure 2. Stages in the life cycle of *Naegleria*. (A) trophozoite. The arrow indicates the single nucleus with its large, central nucleolus. (B) cyst. The arrow indicates the location of a pore in the cyst wall. (C) flagellate. All three stages are shown at the same magnification (x1250) and were photographed using a PM-10AD Photomicrographic System on an Olympus Vanox microscope.

A



B

C

spiny acanthapodia while those of *Naegleria* are limax or slug–like in appearance. *Naegleria* trophozoites show smooth, hemispherical eruptions at their "anterior" ends which alternate from side to side during the advance of the trophozoites. Pseudopodial eruptions are neither smooth nor necessarily anterior during the advance of *Acanthamoeba* trophozoites. Griffin (1978) showed that, in *Naegleria*, both the nucleolus and the nuclear membrane remain intact during mitosis, while, in *Acanthamoeba*, they both disappear. Although amoeba generally lack centrioles some species of *Acanthamoeba* were found to contain centriolar equivalents (Griffin, 1978). Recently, the presence of sucker–like structures on trophozoites of *N. fowleri*, termed "amoebastomes" have been reported (Marciano–Cabral and John, 1983; John *et al*., 1984).

Cysts of *Acanthamoeba* are larger (9–30 um) than those of *Naegleria* (7–21 um). They possess two layers, an outer ectocyst, and an inner endocyst. These layers may show various degrees of wrinkling which often produces a stellate or " star–like" effect as seen in *A comandoni* and *A astronyxis* (Sawyer and Griffin,1971). Some species produce cysts with relatively smooth walls. Trophozoites excyst through pores found at points where the cyst walls overlap. Cysts of *Naegleria* are generally smooth and round. The cysts have a thick inner and a thin outer wall in *N. australiensis* (De Jonckheere, 1981) and *N. gruberi* (Schuster, 1975). The outer wall is not present in cysts of *N. fowleri* and *N. jadini* which may account for the less resistant cysts of the former. Pores have been found in the walls of all species of *Naegleria* examined. Trophozoites emerge when mucoid plugs, found in the pores, are

digested. In *N. australiensis* and *N. gruberi* the pores are surrounded by a collar or rim, which makes them more visible. *N. gruberi* has the ability to synthesize a second cyst if the integrity of the first, outer cyst is disrupted. In reporting this phenomenon Chiovetti (1976) suggested that it was related to a protective mechanism for survival in *Naegleria*. Werth and Kühn (1967) reported that the cyst wall in *N. gruberi*, like that in *Acanthamoeba* (Neff *et al.*, 1964) has a relatively high protein content and both tightly bound and readily extractable lipid components. An alkali–insoluble material in the cyst wall, which appears to be cellulose, may be bound to a lipoprotein.

Mattar and Byers (1971), using *A castellani*, found that a pH of 7 and a temperature of 30° C were optimal activating conditions for excystment. Protein and RNA inhibitors such as cycloheximide and actinomycin D, respectively, inhibit both activation and emergence. In *N. gruberi*, excystment was activated by increased carbon dioxide concentration, proline addition and an increase in the cyst population density (Averner and Fulton, 1966).

The flagellate stage in *Naegleria* is induced by a number of conditions including contact with water and dilution of the culture media (Fulton, 1970a; Woodworth *et al.*, 1982). Fulton (1970b) found that flagellates of *Naegleria*, unlike those of the amoebo–flagellate *Tetramitus rostratus*, could neither feed nor reproduce. Although the majority of *Naegleria* flagellates possess only two flagella, 5–15 % of any flagellate population may possess 1, 3 or 4 flagella, and it is not uncommon for a cell to possess as many as 5 or 6 flagella (Fulton and Dingle, 1967). Heat shocking at 38.2° C was found to produce as many as 28

flagella on a single cell. Dingle (1979) found that pH has a secondary effect on the development of additional flagella by heat-shocked cells. Enflagellation is inhibited by protein inhibitors such as cycloheximide (Woodworth *et al.*, 1982) and puromycin, RNA inhibitors such as actinomycin D (Yuyama,1971) and polymixin B, either alone or with puromycin or actinomycin D (Preston and O'Dell, 1971). Calcium was also found to have both an indirect and a direct inhibitory role in the flagellation of *Naegleria* (Schuster and Twomey, 1983). Griffin's (1983) "flagellate-empty habitat" hypothesis postulates that human intervention or natural events remove natural competitors and the ability to transform to a motile flagellate confers an advantage in recolonizing.

B. Taxonomy and Physiology/Metabolism

Species designation in *Acanthamoeba* is based on complex criteria including growth characteristics, cytopathic effect in culture (CPE) and virulence in mice (De Jonckheere, 1980; Stevens and O'Dell, 1974). The latter characteristic is not as helpful as it is in the case of *Naegleria* infections. Several species of *Acanthamoeba* cause a fatal granulomatous amoebic encephalitis (GAE), including *A castellanii, A culbertsoni, A polyphaga* and *A astronyxis*. Antigenic characteristics expressed through immunofluorescence (IF) (Willaert and Stevens, 1976) and immunoelectrophoresis (IE) (Willaert *et al.*, 1978) are also used to differentiate strains. Protein distribution patterns, obtained through disc electrophoresis, are an additional method of strain identification (Visvesvera and Balamuth, 1975; and Visvesvera *et al.*, 1983).

Five species of *Naegleria* are generally recognized: *N.*

fowleri, the causative agent of PAM; *N. gruberi*; *N. jadini*, considered to be intermediate between *N. fowleri* and *N. gruberi* (Griffin, 1978); *N. lovaniensis*, a high temperature tolerant, non-pathogen; and *N. australiensis sp. n.*, of variable pathogenicity (De Jonckheere, 1981). Species identification is based on a number of factors including growth characteristics (De Jonckheere, 1977), temperature tolerance, virulence in mice (Carter, 1970; De Jonckheere, 1979a), and CPE in culture (Marciano-Cabral and Bradley, 1982; Marciano-Cabral *et al.*, 1982). Antigenic characteristics (De Jonckheere and Van de Voorde, 1977; De Jonckheere *et al.*, 1974; Visvesvara and Healy, 1975) and protein patterns, obtained through disc electrophoresis (Nerad and Daggett, 1979) or iso-electric focusing (IEF) (De Jonckheere, 1981; 1982a; Pernin *et al.*, 1983) were also valuable in species designation. De Jonckheere and Dierickx (1982) found that determination of the specific activity of acid phosphatase (AP; E.C. 3.1.3.2) and leucine amino peptidase (LAP; E.C. 3.4.11.1) allowed for quick identification of *N. fowleri*.

Environmental isolation of members of the genera *Acanthamoeba* and *Naegleria* usually involves their initial concentration on filters (Chang, 1971). Culture onto non-nutrient agar with living or dead bacteria, or into simple or complex media is then required. Partially or completely, defined media have also been used (Fulton, 1974; Ingalls and Brent, 1983; Nerad *et al.*, 1983). Because prolonged axenic cultivation results in loss of virulence (De Jonckheere, 1979a), pathogenic amoebae are often maintained with cells in tissue culture or through serial passage in animal systems. Virulence is restored by using the two preceeding methods as well as by growing amoebae with bacteria. *Naegleria* require more complex axenic

media than *Acanthamoeba* with each species having very critical requirements (Band and Balamuth, 1974; Cline *et al*, 1983). De Jonckheere (1977) has found an elegant medium for differentiating between pathogenic and non-pathogenic isolates of *N. fowleri*.

Pathogenic strains of amoebae are able to tolerate higher temperatures than non-pathogenic strains. Strains of *N. fowleri*, which produce PAM in humans, are able to survive at 45–46° C when growing on *Escherichia coli*, while *A culbertsoni* is able to survive at 42° C under the same conditions (Griffin, 1972). Less virulent strains of *Acanthamoeba* are able to tolerate lower maximum temperatures. Some of these strains are associated with cases of corneal ulcers in humans (Bos, 1981) since, the cornea is, presumably, generally cooler than the brain. Exceptions include *N. lovaniensis*, which grows at 45° C, on *E. coli*, but is non-pathogenic, and *N. australiensis*, which grows at 43° C, under the same conditions, and is of variable pathogenicity.

As mentioned earlier, *N. fowleri* moves much faster than *A culbertsoni* in media approximating conditions in the human body (Griffin, 1978). It has been observed by Culbertson to "outrun" chasing leukocytes which are able to surround and partially destroy *A culbersoni* trophozoites.

C. **Human Infections**

Naegleria meningoencephalitis (PAM) exhibits a standard pattern of infection, pathology and outcome. Infections occur in healthy, young and generally, active persons with a history of exposure to water. Trophozoites or flagellates enter the nose and penetrate the olfactory mucosa, where they proliferate. They pass through the cribriform plate and make their way up the

olfactory nerve to the brain. Penetration of brain tissue, destruction of brain cells and inflammation result. Trophozoites accumulate in the spaces around blood vessels where, it is assumed, the vessels provide an oxygen source for the aerobic pathogens. A more detailed description of the pathology of PAM is provided by Carter (1972). The prognosis of this type of infection is death in all except the rare case.

No set pattern is observed in *Acanthamoeba* infections. GAE is often, but not always, seen in individuals whose immune systems have been impaired. The patient has either been receiving immunosuppressive therapy or was suffering from a chronic or debilitating illness. Trophozoites or cysts enter through lesions in the skin, the eye or the nose (Martinez, 1980; 1982). The trophozoites generally make their way to the target organs through the circulatory system and unlike PAM, where only the brain is involved, GAE may affect the skin, liver, lungs, kidneys, adrenals, pancreas and lymph nodes. Martinez's (1983) review of the pathology of GAE infections should be referred to for further details. The outcome of GAE is less likely to be fatal and in many cases it is suspected that spontaneous remissions have occurred or that a chronic infection persists.

Diagnosis of *Acanthamoeba* infections includes the use of serological procedures, brain needle biopsy, skin biopsy and postmortem study. Spinal fluid samples taken from human disease cases have not always shown the presence of amoebae with the required characacteristics. Diagnosis of *Naegleria* meningoencephalitis is complicated by its similarity to bacterial encephalitis. The absence of a large bacterial population in samples of cerebrospinal fluid eliminates the possibility of this type of illness (Culbertson *et al.*, 1968). Low glucose and high protein levels

in cerebrospinal fluid are another indication of PAM (Duma *et al.*, 1971). Lam *et al.* (1982) proposed the use of computed tomography (CT) patterns as a method for the early detection of PAM, in cases of presumptive bacterial meningitis, when bacteria are not found in the cerebrospinal fluid.

Drug treatment against *Acanthamoeba culbertsoni* infections was most effective when given early after infection. Sulfadiazine was the most effective of three sulphonamides tested *in vitro* (Rowan-Kelly *et al.*, 1982). Polymyxin B sulfate and pentamidine isethionate were also somewhat effective against pathogenic *Acanthamoeba* (Duma and Finley, 1976). Ferrante *et al.* (1984) found that *A culbertsoni* showed a marked sensitivity to colistin (polymyxin E). Treatment of *Naegleria* meningoencephalitis is also hampered by delays in diagnosis. Only after patients demonstrate observable neurological abnormalities such as seizures, coma, ataxia or bizarre behaviour are they brought to a physician's attention. The only patient who survived PAM was a child, diagnosed unusually early in the course of the infection, and treated with amphotericin B (Apley *et al.*, 1970). Amphotericin B was found to be more effective than amphotericin B methyl ester against *N.fowleri* both *in vitro* or *in vivo* (Ferrante, 1982). Imidazole compounds, including miconazole, R41,400 (Ketoconazole) (Elmsly *et al.*, 1980), metronidazole (Flagyl) (Carter, 1969) and clotrimazole (Duma and Finley, 1976) were more effective against *N. fowleri* when tested *in vitro* than *in vivo*. Trimethoprim (2,4-diamino-5-(3,4,5-trimethoxybenzyl)-pyrimidine) has been inhibitory *in vitro* to non-pathogenic *N. fowleri* strains although it did not have any effect on pathogenic strains even at the highest concentrations tested (Cerva, 1980). Δ^9-THC (Δ^9-

Tetrahydrocannabinol provides only minimal protection for mice inoculated with *N. fowleri* (Pringle *et al*., 1979). This drug inhibits the growth of *N. fowleri in vitro* through retarding the synthesis of protein, RNA and DNA (Pringle and Bradley, 1981). Other drugs tested have shown only minimal inhibitory effect against *N. fowleri* (Carter, 1969; Das, 1971).

D. Epidemiology

Freshwater and soil amoebae are ubiquitous. It is not surprising, therefore, that amoebae of the genera *Acanthamoeba* and *Naegleria* are found wherever they are studied: Antarctica, Australia, Belgium, Canada, Czechoslovakia, France, Great Britain, India, Italy, Japan, New Guinea, New Zealand, Nigeria, Northern Ireland, Panama, Puerto Rico, and the United States. Cases of GAE and PAM, world-wide, are reviewed by Griffin (1978), John (1982) and Martinez and De Jonckheere (1981). In the United States, the following states have reported cases of GAE and PAM: Florida, Texas, California, New York, South Carolina, Arizona, Utah, Mississippi, Louisiana, Georgia, Pennsylvania, Virginia and Arkansas.

Acanthamoeba and *Naegleria* have been found in natural environments including soils and leaf litter in Michigan (Umeche, 1983), soil and freshwater in Antarctica (Brown *et al*., 1982) and brackish and ocean sediments (Sawyer *et al*., 1977).

Man-made environments also contain amoebae. Areas in which water has been heated, naturally or artifically are especially likely to contain *Acanthamoeba* and *Naegleria*. Cooling towers and discharge waters from electrical power generating plants in: France (Delattre and Oger, 1981; Dive *et al*., 1981), Czechoslovakia (Cerva *et al*.,1982), West Germany (Janitschke *et al*., 1983; Janitschke *et al*., 1982) and

Belgium (De Jonckheere *et al.*, 1975) are known to contain these amoebae. In the United States power plants in the following states have contained *Acanthamoeba* and *Naegleria*: Florida, Texas (Stevens *et al.*, 1977), Tennessee (Tyndall *et al.*, 1978) and Pennsylvania (Sykora *et al.*, 1983). De Jonckheere (1978a;b) found that industrial effluents, such as those from metallurgic factories contained pathogenic *N. fowleri*. Scaglia *et al.* (1982; 1983) found pathogenic *Naegleria* in swimming pools and mud samples in Italian spas. Brown *et al.* (1983) found pathogenic *Acanthamoeba* and *Naegleria* in thermal areas in New Zealand. Indoor swimming pools in Czechoslovakia (Kadlec *et al.*, 1978) and Belgium (De Jonckheere, 1979b) contained pathogenic amoebae. De Jonckheere's (1979c) review provides a detailed account of the incidence of pathogenic amoebae in swimming pools. Sewage treatment plants are known to contain *Naegleria*, as shown by Singh and Das, in India (1972) and Seyfried *et al.*, in Canada (1983). Even such seemingly innocuous areas as aquaria (De Jonckheere, 1979d) and laboratory waterbaths (Cotter, 1973) contained *Acanthamoeba* and *Naegleria*.

Chlorination is the most widely used treatment for waters suspected of containing pathogenic amoebae. Chlorine has been found to be more efficient in eliminating cysts of either non-pathogenic *N. gruberi* or pathogenic *N. fowleri* than any species of *Acanthamoeba* (De Jonckheere and Van de Voorde, 1976). Chlorinated cyanurates have no measureable cysticidal effect on *N. gruberi*. This is due to the inhibition of the effect of chlorine by cyanuric acid (Engel *et al.*, 1983; Rubin *et al.*, 1983). Cursons *et al.* (1980) found that of four disinfectants studied, Deciquam 222 (de-decyldimethyl-ammonium bromide) was the most effective

amoebocide, followed by chlorine, chlorine dioxide and ozone. Deciquam 222 was twenty times more effective than chlorine when tested at the same concentrations (mg per liter^{-1} and cm^3 per liter^{-1}, respectively). Other halogens found effective in eliminating amoebae include bromine and iodine (Chang, 1978). In comparing swimming pools disinfected with halogens and those irradiated with ultraviolet light, De Jonckheere (1982b) found that thermophilic *Naegleria* were never found in pools using the former, while they were always found in pools using the latter. An alternative to chlorine–based disinfectants, especially in home pools, Baquacil, was able to kill *Acanthamoeba* and *Naegleria* and could be used for decontamination of water containing pathogenic amoebae (Dawson *et al*., 1983a; 1983b).

In 1980 Cotter and Winner (1981) began a study on three power generating stations located on Lake Huron, the Detroit River and Lake Erie (Figure 3). The results they had obtained by the end of 1981 are presented in Table 1. Since consistent positive samples containing potentially pathogenic high temperature tolerant amoebae (amoebae that could survive at 45° C) were obtained only at the J. C. Keith Station the present study continued sampling at this location. In order to determine whether sewage treatment plants in Essex County might harbor pathogenic *Acanthamobae* and *Naegleria*, a primary and a secondary waste treatment plant, in close proximity to the power generating station, were selected for sampling. Waste stabilization ponds at Anderdon and Kingsville were also studied to determine whether they might serve as reservoirs for potentially pathogenic amoebae. Twelve recreational beaches in Essex County were also sampled to determine whether pathogenic amoebae might present a possible hazard to individuals using the beaches. An

Figure 3. Map showing the location of the three power generating plants on the Great Lakes referred to in this study; the Bruce Nuclear station on Lake Huron,the J. C. Keith station on the Detroit River, and the Nanticoke station on Lake Erie.

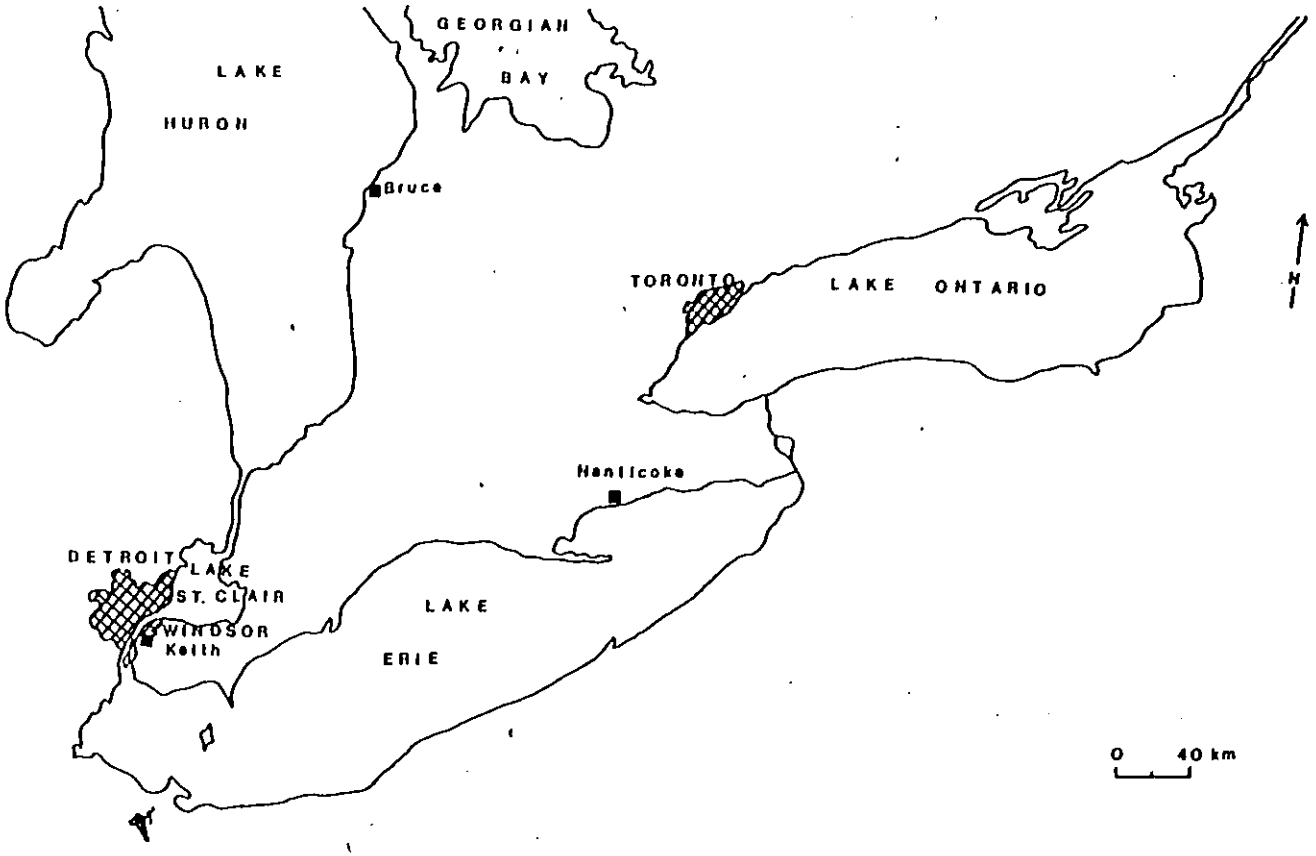

GEORGIAN
BAY
LAKE
HURON
Bruce
TORONTO
LAKE ONTARIO
N
Hanlicoke
DETROIT
LAKE
ST. CLAIR
LAKE
WINDSOR
Keith
ERIE
0 40 km

Table 1:
High Temperature Tolerant Amoebae at Selected Great Lakes Power Generating Plants

Plant	Number Samples Taken	Percentage Positive Samples
Bruce Nuclear, Douglas Pt., Lake Huron		
June - October (1980)	60	0.00
April - October (1981)	70	0.00
J. C. Keith, Windsor, Detroit River		
May - October (1980)	60	5.00
March - October (1981)	144	6.25
Nanticoke, Nanticoke, Lake Erie		
June - October (1980)	60	3.33
April - November (1981)	84	0.00

The values Presented in this table were calculated from data provided by Cotter and Winner (1981)

additional purpose in sampling the beaches was to determine whether thermal or fecal pollution, from the power generating station or the waste treatment plants (and waste stabilisation ponds), respectively, had any effect on populations of potentially pathogenic amoebae located at these sites.

MATERIALS AND METHODS

A. Sampling Procedures

All water samples were taken using sterilized sampling bulbs and 300 milliliters were emptied into sterile polyvinyl chloride Whirl-Pak (Nasco) bags. Ten grams of sediment were placed in the same types of bags for subsequent analysis. All samples were kept in an ice-filled styrofoam container. prior to delivery back at the laboratory. No samples were kept on ice for more than six hours prior to being analyzed.

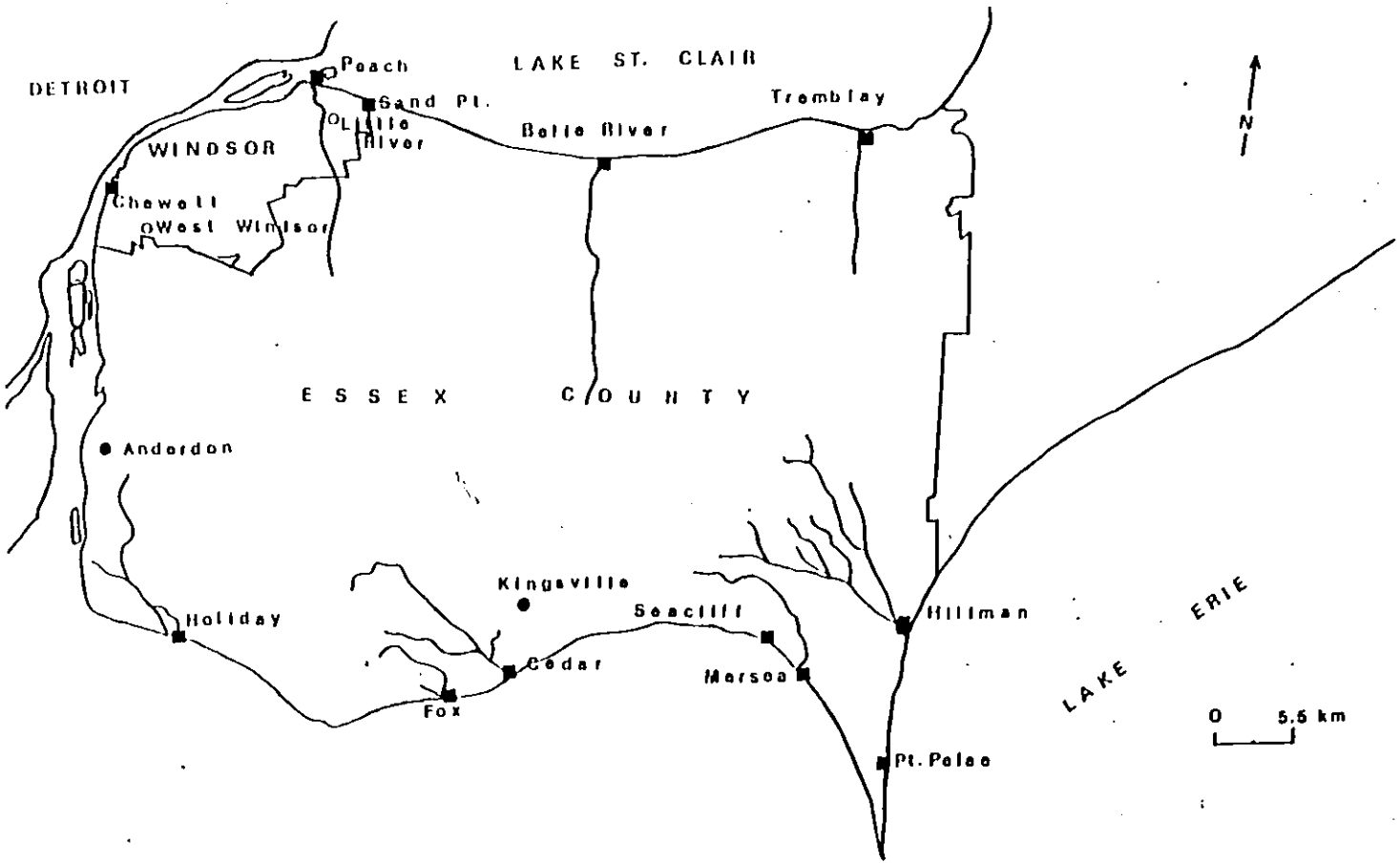
1. Recreational Beaches

Twelve beaches in Essex County were sampled in 1982 and eleven in 1983 (Figure 4). At each beach sampling was done as follows. In 1982 two sites were sampled: (A) at a point 1 meter off shore a surface water sample was obtained as well as a sediment sample immediately underlying the water sample, and (B) 5 meters off shore water samples were taken at the surface as well as at a depth of 1 meter.

In 1983 sampling was eliminated at Peach Island due to the scarcity of positive samples obtained from this location as well as to the difficulty in obtaining these samples. Because samples taken from site A provided an adequate reflection of the presence of high temperature tolerant amoebae at each beach, sampling at site B was eliminated. A single surface water and underlying sediment sample were taken 1 meter off shore at two locations approximately 10. meters apart (designated I and II). Percentages of positive amoeba samples were calculated on the basis of either the three water and one sediment samples obtained at each beach, in 1982, or the two water and two sediment samples obtained at each beach, in 1983. Additional water samples were taken and analyzed on site for pH and dissolved oxygen content. In 1983

Figure 4. Map showing the location of the twelve recreational beaches sampled in Essex County , as well as two waste treatment plants and waste stabilization ponds .

(■) recreational beaches;
(○) waste treatment plants;
(●) waste stabilization ponds.


DETROIT
Peach
LAKE ST. CLAIR
Sand Pt.
Little River
Belle River
Tremblay
WINDSOR
Chewett
West Windsor
N
ESSEX COUNTY
Anderdon
Kingsville
Holliday
Seacliff
Hillman
Cedar
Mersea
Fox
LAKE ERIE
Pt. Pelee
0 5.5 km

the pH was not measured although both the air and water temperature were taken during the two years of the study.

2. J. C. Keith Power Generating Plant

Water samples were obtained from this plant at three sites: (A) the point of entry of thermally enhanced water into the lake (shore site), (B) a point 50 meters off shore, and (C) a point 100 meters off shore. A sample was taken immediately above the bottom sediment, at a point mid-way between the previous sample and the surface, and at the surface of each site. A total of nine water samples were collected every two weeks for microbiological analysis. Additional water samples were obtained and analyzed directly on site for pH and dissolved oxygen content. The temperature of each water sample was measured and a single measurement of the air temperature at the shore site was made.

3. Chemical Waste Treatment Plants

Samples were obtained from one primary and one secondary waste treatment plant in the vicinity of the J. C. Keith Power Generating Plant. The Little River Plant is a secondary waste treatment plant which utilizes aerobic bacteria to break down raw sludge prior to chlorination. At this plant samples were taken at each step in the treatment process during 1982. Raw sludge, activated and return activated sludge, as well as unchlorinated and chlorinated effluent samples were obtained. In 1983, activated and return activated sludge samples were omitted. At the West Windsor Plant the aeration process is not performed in the presence of aerobic bacteria since the waste received by this plant is basically industrial and chemical in nature. Such waste would result in the death of the bacteria normally used in this process.

Therefore during 1982 and 1983 only raw sludge, unchlorinated effluent and chlorinated effluent samples were obtained from this plant.

4. Waste Stabilisation Ponds

A number of communities in Essex County use waste stabilisation ponds (oxidation ponds or sewage lagoons) for treatment of waste materials. During 1982 three ponds in Anderdon were sampled. Water and sediment samples were taken from each pond every two weeks. In 1983 only Pond 1 was sampled although additional samples of the effluent from the pond were obtained. Of the three ponds at Kingsville only Pond 2 was sampled during 1982 and 1983. Water and sediment samples were taken from the pond. The effluent common to all three ponds was also sampled.

B. Measurement of Physical, Chemical and Biological Parameters

1. Air and Water Temperature

A thermometer was used to measure the temperature after two to three minutes of acclimation in the air or at the water level required.

2. pH

The Hach Chemical Test Kit colorimétric test was used to determine the pH.

3. Dissolved Oxygen

The dissolved oxygen content in parts per million was measured using a Hach Chemical Test Kit and/or a dissolved oxygen meter (YSI Model 51B).

4. Total Bacteria

Water samples were diluted and added to quadruplicate plates of Tryptic Soy agar (Difco). An inoculum of 0.1 ml was spread evenly over the plate using a sterile glass rod which had been bent to form a 90–degree angle. Plates were then incubated in an inverted position at 37° C for twenty–

four hours. Total bacterial numbers per milliliter were then determined.

5. **Fecal Coliforms**

Fecal coliform numbers were determined by filtering 100 ml of water sample through a 0.8 um HCGW sterile filter (Millipore) and incubating in a petri dish containing FC broth at 44.5° C for eighteen to twenty-four hours. Numbers were always reported per 100 ml.

C. Isolation and Identification of Amoebae

1. **Isolation of *Acanthamoeba* and *Naegleria***

Recovery of high temperature tolerant amoebae from water and sediment samples was based on previous experience as well as reviews by (Chang, 1971; Singh and Das, 1972). The medium used to recover amoeba was buffered 0.1 % lactose peptone agar (LP) (1 gm lactose; 1 gm Bacto–peptone; 15 gm Bacto–agar; and 1 liter of potassium phosphate buffer, 5 mM at pH 7.0). The autoclaved medium was then dispensed in 10 ml quantities into 15 cm diameter petri dishes (Fisher). The host bacterial strain used to culture amoebae was *Escherichia coli* strain B/r.

Since early experiments had indicated that amoebae were present in low numbers at recreational areas, a semiquantitative method of recovery was used as a standard. One hundred milliliters of the water sample were filtered through a sterile 0.45 um filter and then the filter was placed face up on a plate of buffered 0.1 % LP agar. Each plate was then washed with four to five milliliters of a mixture of one hundred milliliters of sterile double distilled (SDD) water containing a loopfull of an overnight culture of *E. coli* B/r. The bacteria had been grown on glucose salts (GS) (1.0 gm NH_4Cl; 0.13 gm $MgSO_4$; 3.0 gm KH_2PO_4; 6.0 gm Na_2HPO_4; 20.0 gm Difco agar; 1 liter double distilled water; 10.0

ml of 0.4 gm/ml sterile glucose). The "flooded" cultures were incubated upright at 41° C and examined daily for eleven consecutive days for the presence of amoebae. Plates which failed to show amoeba growth after this period were discarded.

Plates containing amoebae were subcultured as follows. The plates were scraped with a bent sterile glass rod so as to loosen amoebae from the agar. A homogeneous suspension of 0.5 to 1.0 ml was placed on a plate of non-nutrient medium (NNM) agar (15 gm Bacto-agar; 1 liter potassium phosphate buffer, 5 mM at pH 7.0) and a plate of 0.1 % LP agar containing 1.0 % NaCl, for the isolation of *Naegleria* and *Acanthamoeba*, respectively. Additional SDD water, four to five milliliters, was added to each of the plates. The plates were then incubated at 44.5° C, for *Naegleria*, and 41° C, for *Acanthamoeba*.

Plates of NNM and 0.1 % LP with NaCl agar showing vigorous growth of amoebae were examined using phase-contrast microscopy for the presence of amoebae and cysts with the characteristics of *Naegleria* and *Acanthamoeba*, respectively. A flagellation test was performed on plates containing presumptive *Naegleria* as follows: an overnight plate of amoebae grown at 37° C with a minimal amount of culture liquid (two to three milliliters of SSD water and *E. coli* B/r) was flooded with five milliliters of SDD water and incubated at 37° C. From two hours up to four hours later slides were made and examined under phase-contrast microscopy for the presence of flagellates characteristic of *Naegleria*. Selected flagellates were monitored for transformation back to the amoeboid form. No further testing was done on plates containing *Acanthamoeba*.

2. Identification of *Naegleria*

(a) Growth in Serum–Casein–Glucose–Yeast Extract Medium (SCGYEM). *Naegleria* isolates were freed from the majority of bacterial contamination by one of the following methods. The isolates were subcultured onto plates of NNA containing antibiotics (400 U/ml Penicillin–G; 200 ug/ml Streptomycin–sulphate; 200 ug/ml Neomycin–sulphate) with live *E. coli* B/r and the cultures were incubated that 37° C for two to three days or the amoebae were fed autoclaved *E. coli* B/r which had been grown in 0.1 % LP broth and cultured on NNA plates at 41° C or 44.5° C.

In 1982, the amoebae were inoculated directly into tubes of SCGYEM and incubated at 37° C (De Jonckheere, 1977). Rapid growth in this axenic medium is indicative of pathogenic *Naegleria* and *Acanthamoeba* species (De Jonckheere, 1977, 1980). In addition, this medium allows for selective growth of pathogenic strains over non–pathogenic strains in mixed cultures.

Naegleria isolates were cloned, in 1983, so as to produce single strain isolates for inoculation into mice. Triplicate dilutions of *Naegleria* were placed on NNA plates with a heavy inoculum of *E. coli* B/r and the mixture was spread evenly over the plate with a sterile bent glass rod. After incubation at 37° C for twenty–four to forty–eight hours, individual plaques could be seen on the plate as a clearing of the bacterial "lawn". Samples were taken from the edge of three or four well–separated plaques. Each sample was placed on individual NNA plates and tested for flagellation, as described previously, to confirm the presence of *Naegleria*.

(b) Mouse Pathogenicity Test. All positive cultures (amoebae capable of

growing at 44.5° C with the characteristics of *Naegleria*) which could be recovered from storage were used to inoculate mice. Amoebae were washed free of media by differential centrifugation and a suspension of 0.5 x 10^6 to 1.0 x 10^6 amoebae per milliliter was prepared. Each of four or five 3–4 week old CD1 mice were inoculated with 10–20 ul suspensison by intranasal installation (Chang, 1971). Caged mice were kept in a laminar flow safety cabinet (LABGARD) for twenty–one days and provided with food and water during this period. Mice which developed characteristic symptoms were to be sacrificed and autopsied for evidence of meningoencephalitis and the accompanying amoeboid organisms (Singh and Das, 1972b). In order to confirm the absence of amoebae in mice which survived the twenty–one day observation period, random mice were selected, sacrificed and autopsied.

(c) Polyacrylamide Gel Electrophoresis. *Naegleria* isolates were grown on ten to twelve plates of 0.1 % LP and the amoebae were harvested and concentrated. Amoebae were washed free of bacteria and medium by washing three times with phosphate buffer. The pellet was then suspended in one milliliter of buffer and put through three cycles of "freeze–thaw" (frozen at −20° C and thawed at 23.5° C). This crude enzyme extract was then dispensed into 1.5 milliliter Eppendorff tubes in aliquots of 250 ul and stored at −20° C for use in subsequent analysis of isoenzyme patterns.

Standard strains of high temperature tolerant *Naegleria fowleri* (ATCC 30100, ATCC 30472) and *Naegleria lovaniensis* (ATCC 30569) were also grown in SCGYEM medium, washed free of medium in phosphate buffer and "freeze–thaw" extracts were stored at −20° C. Extracts contained 0.6–1.2 x 10^7 amoebae per milliliter.

Native polyacrylamide gels (7.5 %) were prepared using 0.2 M Tris–glycine buffer, pH 8.9 (Anonymous, 1977). A 3.5 % stacking gel, made using the same buffer as above, was poured on top of the separating gel. The electrode buffer was made by diluting one part of the buffer stock solution with one part distilled water. After prerunning the gels at 2 mA per gel for thirty minutes, 50 or 100 ul of the enzyme extract was placed on the gel. The current was run until the dye front (0.25 % Bromphenol blue) reached the bottom of the tube, the gels were removed from the glass tubes and placed into the appropriate staining mixture.

In order to stain for acid phosphatase, 100 mg Na–α-naphthyl acid phosphate and 100 mg Fast Black K Salt were dissolved in 100 ml of 0.05 M acetate buffer, pH 5.0 (Nerad and Daggett, 1979). Gels were placed in the reaction mixture and incubated, at 37° C, in the dark, for one hour or until bands appeared.

The staining mixture for leucine amino peptidase consisted of 20 mg L–leucyl–β–naphthylamide and 25 mg Fast Black K Salt dissolved in 50 ml 0.2 M Tris–maleate buffer, pH 6.0 (Nerad and Daggett, 1979). The gels were allowed to incubate in the reaction mixture, in the dark, at 37° C, until bands became evident.

RESULTS

A. Recreational Beaches

1. Temporal Variation

A general increase in the percentage of samples containing high temperature tolerant amoebae (HTTA), i.e., amoebae capable of growing at 44.5° C, *Acanthamoeba*, and *Naegleria* was evident, over the sampling period, in both 1982 and 1983 (Figures 5A, 5B). Standard deviations for these two figures are presented in the **APPENDIX** (Tables 24 and 25, respectively). Increases were also observed in both air and water temperature (Figures 6A, 6B). Standard deviations for these two figures are presented in the **APPENDIX** (Tables 26 and 27, respectively). In 1982, both bacterial and fecal coliform numbers fluctuated widely, as did fecal coliform numbers in 1983 (Figures 7A, 7B). Standard deviations for these two figures are presented in the **APPENDIX** (Tables 28 and 29, respectively). Bacterial numbers showed a general increase over the sampling period. The pH did not vary significantly during 1982 (Data not shown). Dissolved oxygen content varied greatly and in a random fashion during both years in which it was measured (Data not shown).

The coefficient of correlation (Pearson product moment of correlation) was calculated in order to determine whether there was any statistically significant linear correlation between the percentage of samples containing HTTA or *Acanthamoeba* and the parameters measured (at a level of significance of 95 %). The results are presented in Tables 2 and 3 and confirm that the increases in the percentage of positive samples are related to sampling period, at least in 1982. In 1983, only the percentage of samples containing *Acanthamoeba* was found to be correlated in a positive linear

Figure 5A. Percentages of positive amoebae samples at Essex County recreational beaches during 1982. Each value is an average of the results obtained from four samples taken at each of twelve beaches.

(○) high temperature tolerant amoebae;
(□) *Acanthamoeba*;
(▽) *Naegleria*.

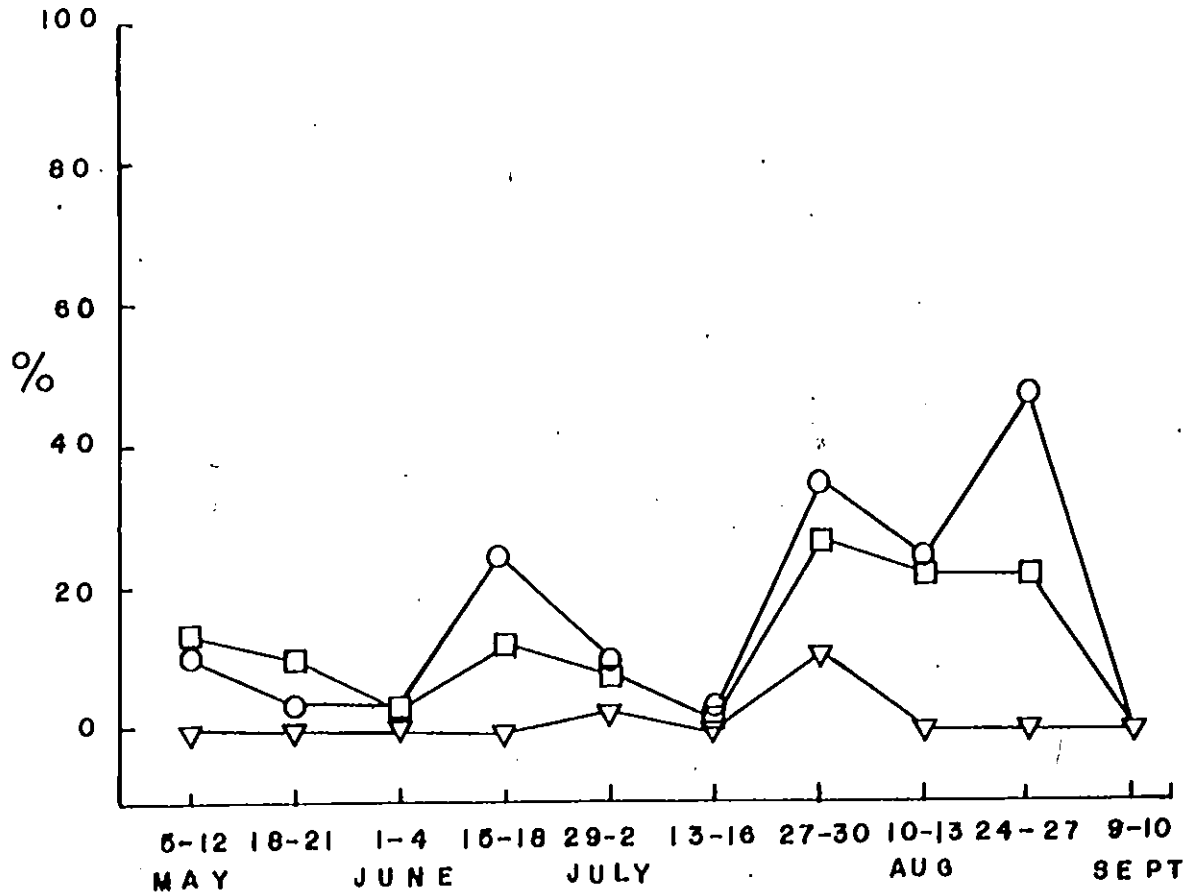

100
80
60
%
40
20
0
5-12 18-21 1-4 15-18 29-2 13-16 27-30 10-13 24-27 9-10
MAY
JUNE
JULY
AUG
SEPT

Figure 5B. Percentages of positive amoebae samples at Essex County recreational beaches during 1983. Each value is an average of the results obtained from four samples taken at each of eleven beache.

(○) high temperature tolerant amoebae;
(□) *Acanthamoeba*;
(▽) *Naegleria*.

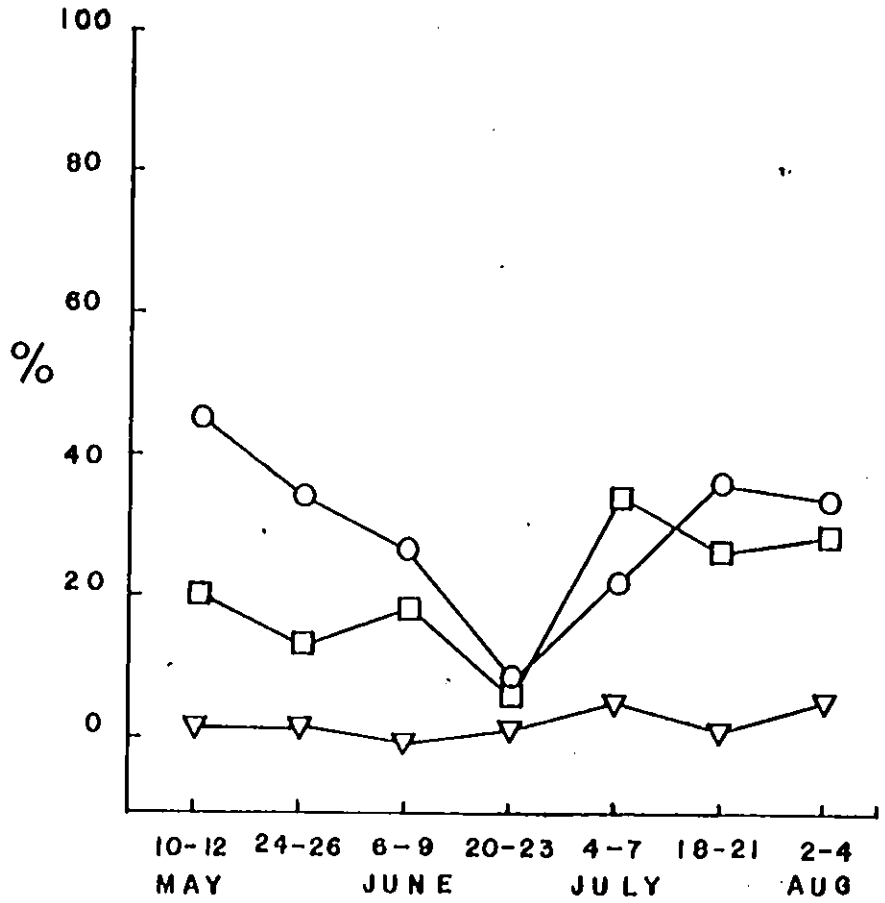

100
80
60
%
40
20
0
10-12
MAY
24-26
6-9
JUNE
20-23
4-7
JULY
18-21
2-4
AUG

Figure 6A. Temperatures measured at Essex County recreational beaches in 1982. The air temperature measurements are averages based on a single measurement taken at each of the twelve beaches sampled. The water temperature measurements are averages based on the single measurements taken at the surface of the water at Site A of each beach.

(□) average air temperature;
(○) average water temperature.

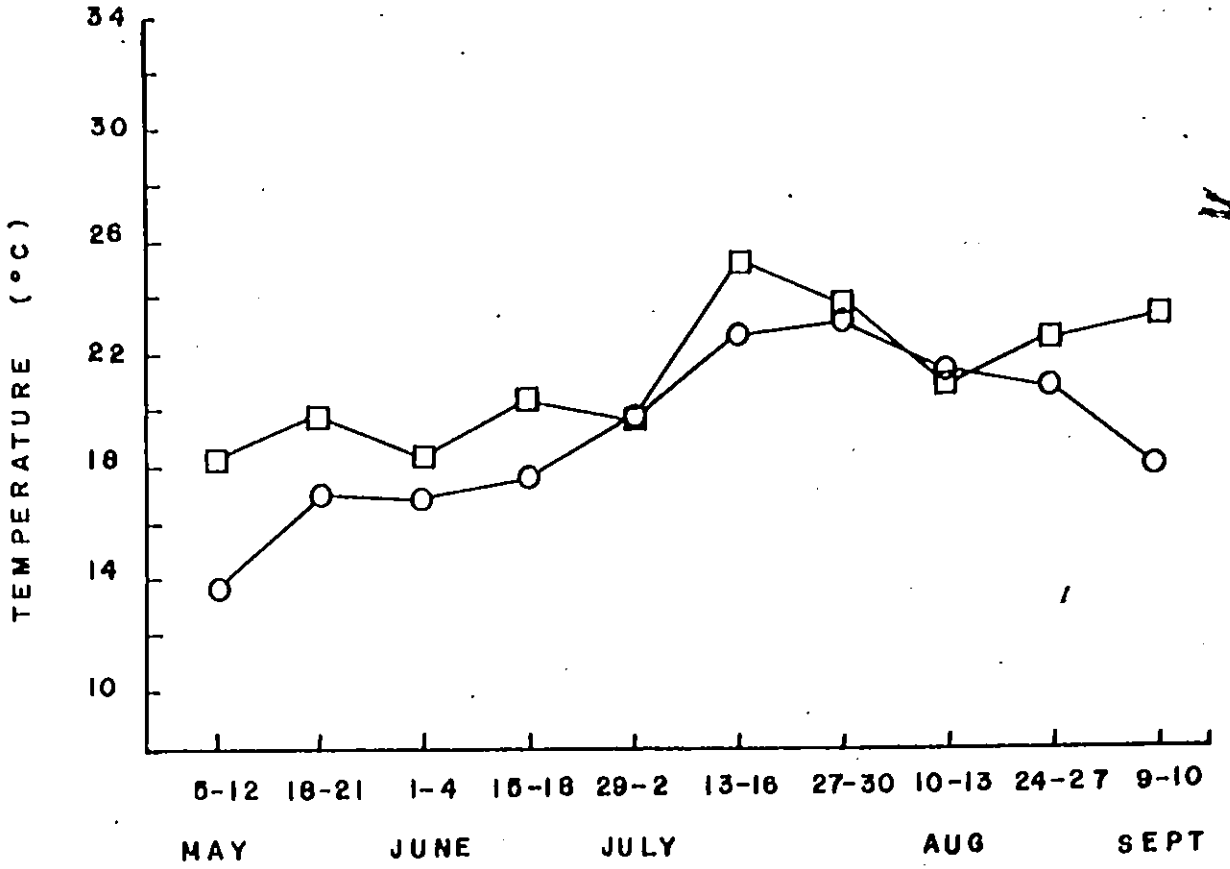

34
30
26
22
18
14
10
TEMPERATURE (°C)
5-12
18-21
1-4
15-18
29-2
13-16
27-30
10-13
24-27
9-10
MAY
JUNE
JULY
AUG
SEPT

Figure 6B. Temperatures measured at Essex County recreational beaches in 1983. The air temperature measurements are averages based on single measurements taken at each of the eleven beaches sampled. The water temperature measurements are averages based on the single measurements taken at the surface of the water at Site A of each beach.

(□) average air temperature;
(○) average water temperature.

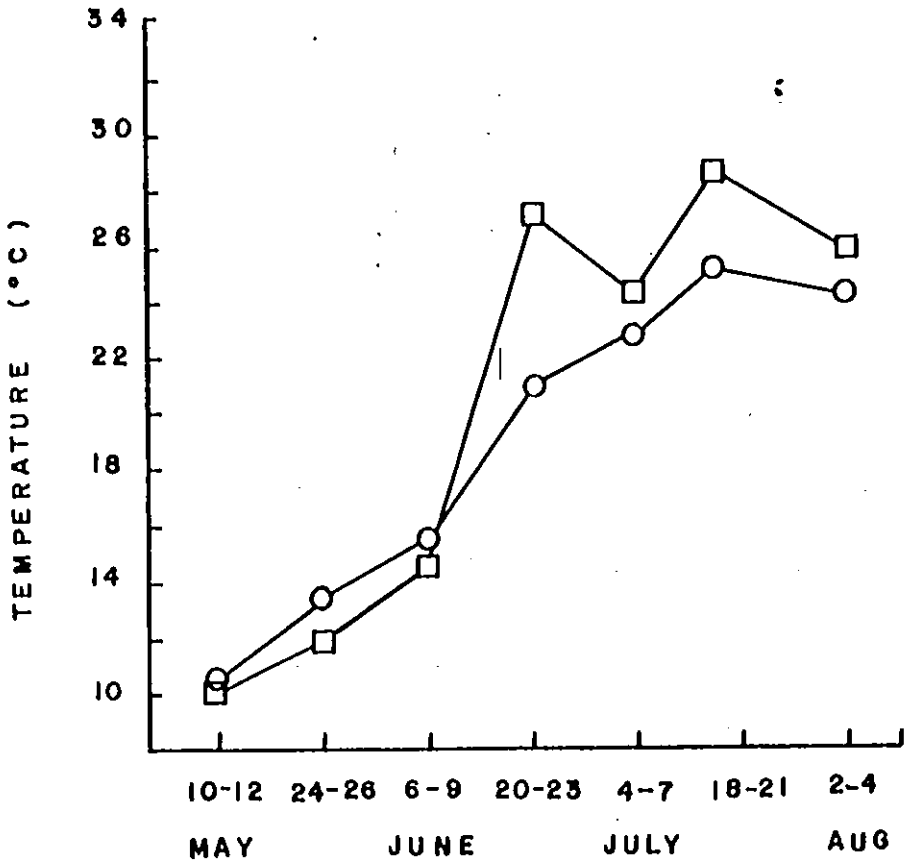

34
30
26
22
18
14
10
TEMPERATURE (°C)
10-12
24-26
6-9
20-23
4-7
18-21
2-4
MAY
JUNE
JULY
AUG

Figure 7A. Average bacterial and fecal coliform numbers measured at Essex County recreational beaches in 1982. Each value plotted is the average of three measurements at each of the twelve beaches sampled.

(●) bacteria per ml;
(○) fecal coliforms per 100 ml.

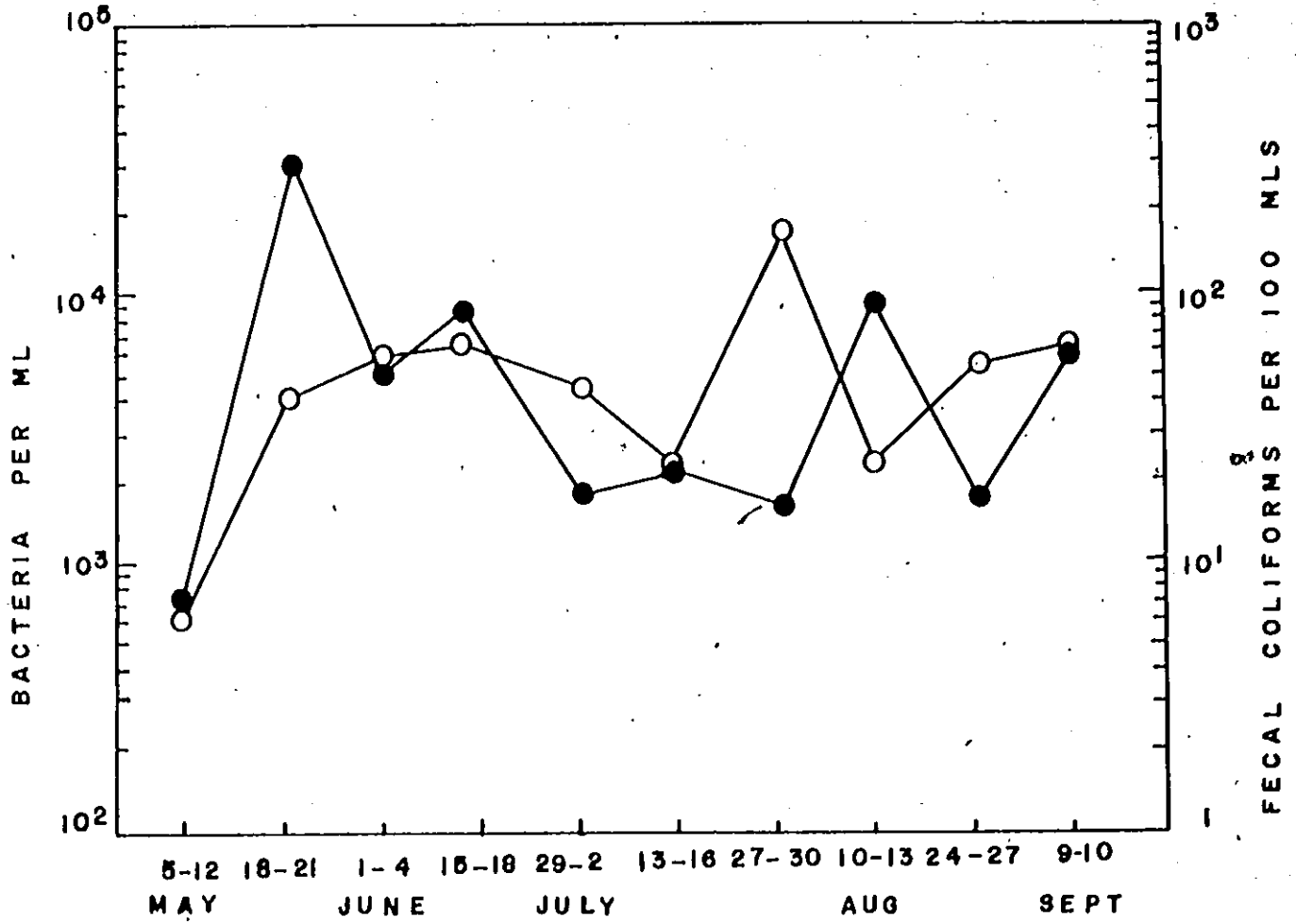

BACTERIA PER ML
FECAL COLIFORMS PER 100 MLS
10⁵
10⁴
10³
10²
10³
10²
10¹
1
5-12 18-21 1-4 15-18 29-2 13-16 27-30 10-13 24-27 9-10
MAY
JUNE
JULY
AUG
SEPT

Figure 7B. Average bacterial and fecal coliform numbers measured at Essex County recreational beaches in 1983. Each value plotted is the average of three measurements at each of the eleven beaches sampled.

(●) bacteria per ml;
(○) fecal coliforms per 100 ml.

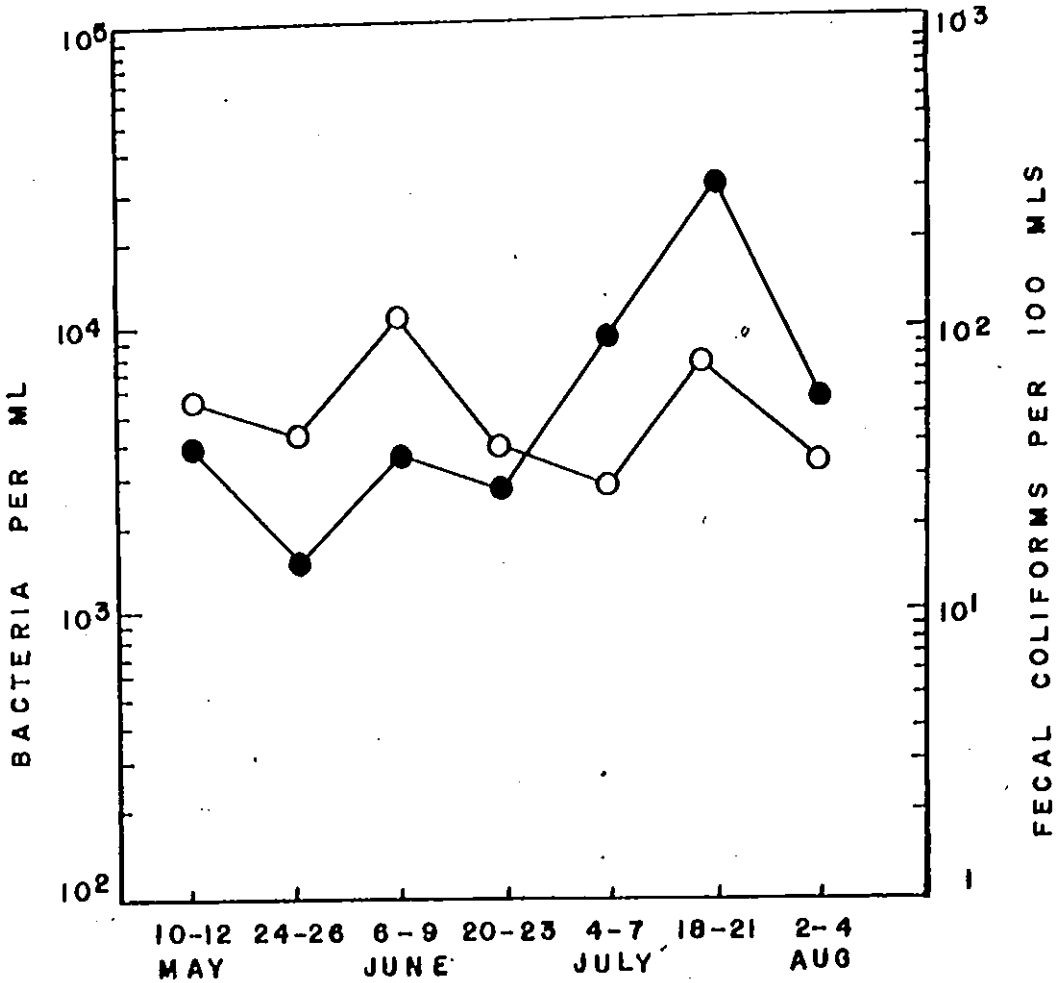

BACTERIA PER ML
10^5
10^4
10^3
10^2
FECAL COLIFORMS PER 100 MLS
10^3
10^2
10^1
1
10-12 MAY
24-26
6-9 JUNE
20-23
4-7 JULY
18-21
2-4 AUG

Table 2:

Pearson Correlation Coefficients for Biological, Chemical and Physical Parameters Measured at Essex County Recreational Beaches (1982).

Parameter	High Temperature Tolerant Amoebae	Acanthamoeba
Sampling Period	0.42401 0.0001	0.27262 0.0040
Dissolved Oxygen	- 0.01540 0.8732	- 0.06487 0.5008
Air Temperature	0.15376 0.1088	0.02298 0.8117
Water Temperature	0.25549 0.0071	0.19291 0.0435
Total Bacteria	- 0.03592 0.7095	0.05569 0.5633
Fecal Coliforms	- 0.00017 0.9986	0.08197 0.3946

The upper number of the pair is the correlation coefficient (r). The lower number is the probability that the null hypothesis of zero correlation is correct. The correlation coefficients are based on 110 observations.

Table 3:

Pearson Correlation Coefficients for Biological, Chemical and Physical Parameters Measured at Essex County Recreational Beaches (1983).

Parameter	High Temperature Tolerant Amoebae	Acanthamoeba
Sampling Period	- 0.06438 0.5780	0.26773 0.0186
Dissolved Oxygen	0.15721 0.1721	- 0.22670 0.0474
Air Temperature	- 0.17769 0.1221	0.19005 0.0978
Water Temperature	- 0.04933 0.6701	0.28767 0.0112
Total Bacteria	0.08297 0.4731	0.13310 0.2485
Fecal Coliforms	0.02199 0.8484	- 0.04387 0.7048

The upper number of the pair is the correlation coefficient (r). The lower number is the probability that the null hypothesis of zero correlation is correct. The correlation coefficients are based on 77 observations.

fashion with sampling period. Because there were so few samples containing *Naegleria*, 4 and 9, in 1982 and 1983, respectively, no attempt was made to correlate the parameters measured with positive samples.

Water temperature was found to be positively correlated with both HTTA and *Acanthamoeba*, in 1982, and with *Acanthamoeba* alone, in 1983, at a statistically significant level (P > 95 %). Air temperature was positively correlated with *Acanthamoeba*, in 1983, at a statistically significant level. No other parameter measured was significantly correlated with either HTTA or *Acanthamoeba*.

2. **Spatial Variation**

Analysis of the individual beaches showed that, in 1982, beaches on Lake Erie had a higher percentage of positive samples containing HTTA, *Acanthamoeba*, and *Naegleria* than did beaches on Lake St. Clair and the Detroit River (Tables 4, 5, 6). Beaches on Lake St. Clair had a somewhat greater or an equal percentage of positive samples, in all three categories, than did the single beach on the Detroit River. In 1983, beaches on Lake St. Clair had the highest percentage of positive samples of HTTA and *Acanthamoeba*. Chewitt Beach, on the Detroit River, had the second highest percentage of positive HTTA and *Acanthamoeba* samples. Only beaches on Lake Erie were found to contain positive *Naegleria* samples during both 1982 and 1983. *Naegleria* were found at Hillman Marsh Beach in both years but only in 1982 at Pt. Pelee Beach. Samples from Fox Creek, Cedar, and Mersea Township beaches were found to contain *Naegleria* only in 1983.

One-way analysis of variance (ANOVA) of positive HTTA,

Table 4:
Analysis of High Temperature Tolerant Amoebae at Essex County Recreational Beaches.

Major Body of Water / Beach	Percentage of Positive Samples 1982[a]	1983[b]
Lake St. Clair	17.15 (±3.30)	38.14 (±7.38)
Trombley	20.00	35.71
Belle River	20.00	46.43
Sand Point	14.29	32.14
Peach Island	14.29	ND
Detoit River	17.14 (±0.00)	35.71 (±0.00)
Chewitt	17.14	35.71
Lake Erie	20.41 (±2.57)	25.51 (±7.27)
Holiday	22.86	10.71
Fox	22.86	21.43
Cedar	17.14	28.57
Seacliff	22.86	28.57
Mersea Township	20.00	28.57
Point Pelee	20.00	28.57
Hillman Marsh	17.14	32.14

[a]The percentage of positive samples at each beach is the average of 35 samples.
[b]The percentage of positive samples at each beach is the average of 28 samples.
ND = Not Done.
The number in parentheses is the standard deviation of the lake or river average.

Table 5:
Analysis of Acanthamoeba at Essex County Recreational Beaches.

Major Body of Water / Beach	Percentage of Positive Samples 1982[a]	1983[b]
Lake St. Clair	11.43 (±5.22)	27.39 (±13.52)
Trombley	17.14	21.43
Belle River	8.57	42.86
Sand Point	14.29	17.86
Peach Island	5.71	ND
Detroit River	11.43 (±0.00)	25.00 (±0.00)
Chewitt	11.43	25.00
Lake Erie	19.18 (±5.40)	19.39 (±5.05)
Holiday	8.57	14.29
Fox	20.00	21.43
Cedar	20.00	21.43
Seacliff	17.14	21.43
Mersea Township	25.71	21.43
Point Pelee	20.00	21.43
Hillman Marsh	22.86	14.29

[a]The percentage of positive samples at each beach is the average of 35 samples.
[b]The percentage of positive samples at each beach is the average of 28 samples.
ND = Not Done.
The number in parentheses is the standard deviation for the lake or river average.

Table 6:
Analysis of Naegleria at Essex County Recreational Beaches.

Major Body of Water / Beach	Percentage of Positive Samples 1982[a]	1983[b]
Lake St. Clair	0.00 (±0.00)	0.00 (±0.00)
Trombley	0.00	0.00
Belle River	0.00	0.00
Sand Point	0.00	0.00
Peach Island	0.00	ND
Detroit River	0.00 (±0.00)	0.00 (±0.00)
Chewitt	0.00	0.00
Lake Erie	1 63 (±3.24)	5.10 (±6.79)
Holiday	0.00	0.00
Fox	0.00	3.57
Cedar	0.00	3.57
Seacliff	0.00	0.00
Mersea Township	0.00	10.71
Point Pelee	2.86	0.00
Hillman Marsh	8.57	17.86

[a]The percentage of positive samples at each beach is the average of 35 samples.
[b]The percentage of positive samples at each beach is the average of 28 samples.
ND = Not Done.
The number in parentheses is the standard deviation for the lake or river average.

Acanthamoeba, and *Naegleria* samples showed no statistically significant difference among the three categories of amoeba at a significance level of 95 % (Table 7). Variation among the samples did not appear to be as great as that within the groups.

3. **Yearly Variation** –

There appeared to a dramatic increase in the percentage of positive samples, in all three categories, from 1982 to 1983. A detailed analysis of the data for HTTA is presented in Table 8. When the data in columns 1 and 3 of this table are compared, only Holiday and Fox beaches appeared to show decreases in the percentage of positive samples as compared to the previous year. The type of samples taken in 1983, however, were different from those taken in 1982, as described in the *MATERIAL AND METHODS* section. When similar types of samples were compared, as found in columns 2 and 3, a decrease in the percentage of positive samples is evident at 8 out of the 11 beaches sampled in 1983. There were increases in the percentage of positive HTTA samples present at Trombley, Cedar and Mersea Township beaches of 17.62, 17.64 and 38.22 %, respectively. At Holiday Beach a dramatic decrease of 194.86 % was apparent in 1983. ANOVAs showed that although there was a statistically significant difference between the population in columns 1 and 3, there was no difference between the populations in columns 2 and 3, when tested at a level of significance of 95 %.

4. **Sample Variation**

Sediment versus Water Samples. Sediment and water samples were taken during the two years of the study. Analysis of this data revealed that sediments contained a much higher percentage of HTTA (Figures 8A, 8B),

Table 7.

One-Way Analysis of Variance for Amoeba Populations With Respect to Location on Major Body of Water.

	F-value	
	1982[a]	1983[b]
High Temperature Tolerant Amoebae	1.93	3.21
Acanthamoeba	0.49	0.97
Naegleria	3.08	1.31
$F_{0.05}$	4.26	4.46

[a]Degrees of freedom = 2 / 9.
[b]Degrees of freedom = 2 / 8.

Table 8:

Impact of Thermal Pollution on the Percentage of Samples Containing High Temperature Tolerant Amoebae at Recreational Beaches in Essex County.

Beach	1982[a]	1982[b]	1983[c]	% Change[d]
Trombley	20.00	29.41	35.71	+ 17.62
Belle River	20.00	52.94	46.83	- 13.05
Sand Point	14.29	35.29	32.14	- 9.80
Peach Island	14.29	23.53	ND	ND
Chewitt	17.14	36.84	35.71	- 3.16
Holiday	22.86	31.58	10.71	-194.86
Fox	22.86	35.29	21.43	- 64.68
Cedar	17.14	23.53	28.57	+ 17.64
Seacliff	22.86	35.29	28.57	- 23.52
Mersea	20.00	17.65	28.57	+ 38.22
Point Pelee	20.00	41.48	28.57	- 44.14
Hillman Marsh	17.14	47.06	32.14	- 41.42

[a]Uncorrected average representing samples taken at Sites A and B.

[b]Corrected average representing samples taken at Site A alone.

[c]Averages representing duplicate samples taken at Site A alone.

[d]% Change = (1983 - 1982[b] / x 1983) x 100 %

ND = Not done.

Figure 8A. Percentages of high temperature tolerant amoebae at Essex County recreational beaches in sediments and water samples (1982). Each sediment value plotted is the average of one sample taken at each of twelve beaches. Each water sample value plotted is the average of three samples at each of twelve beaches.

(●) percent high temperature tolerant amoebae in sediment samples;

(○) percent high temperature tolerant amoebae in water samples.

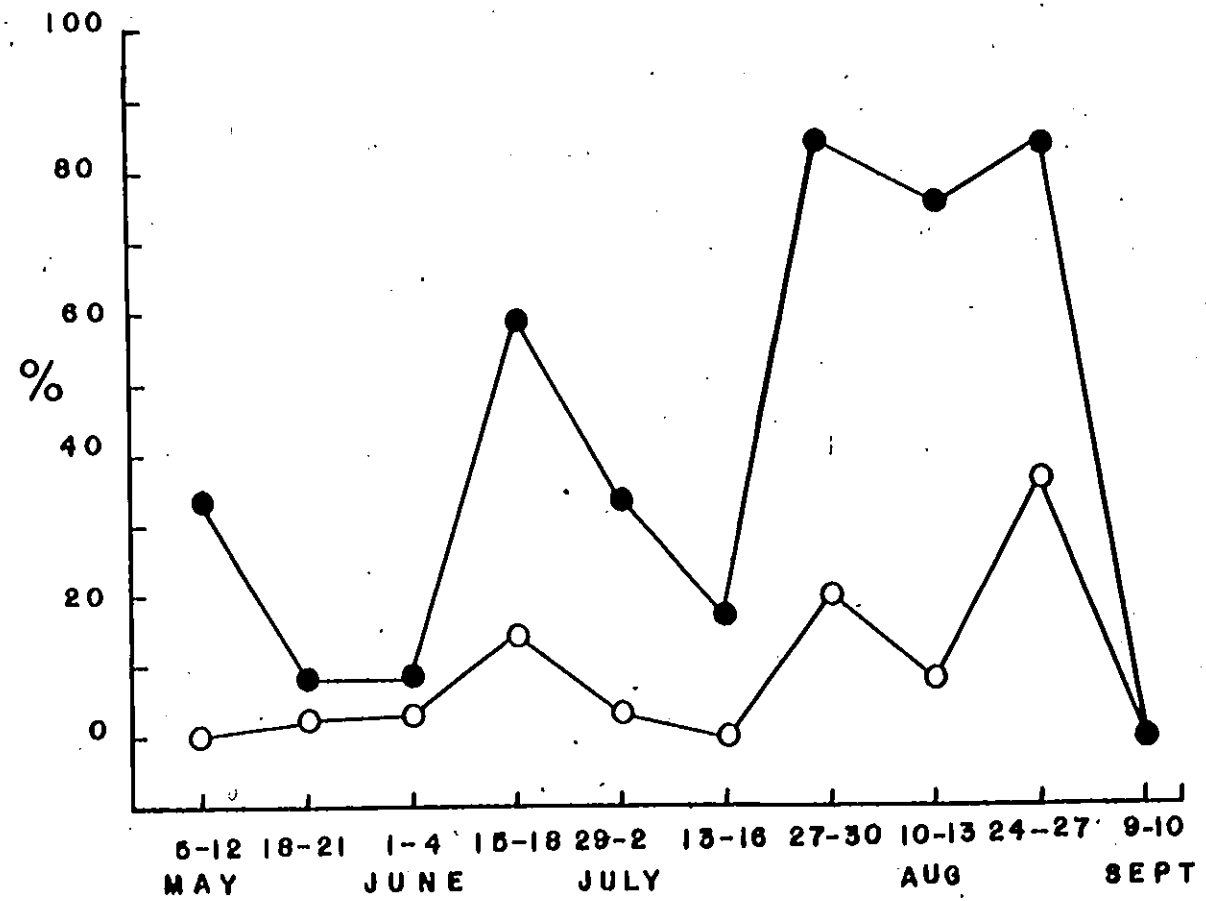
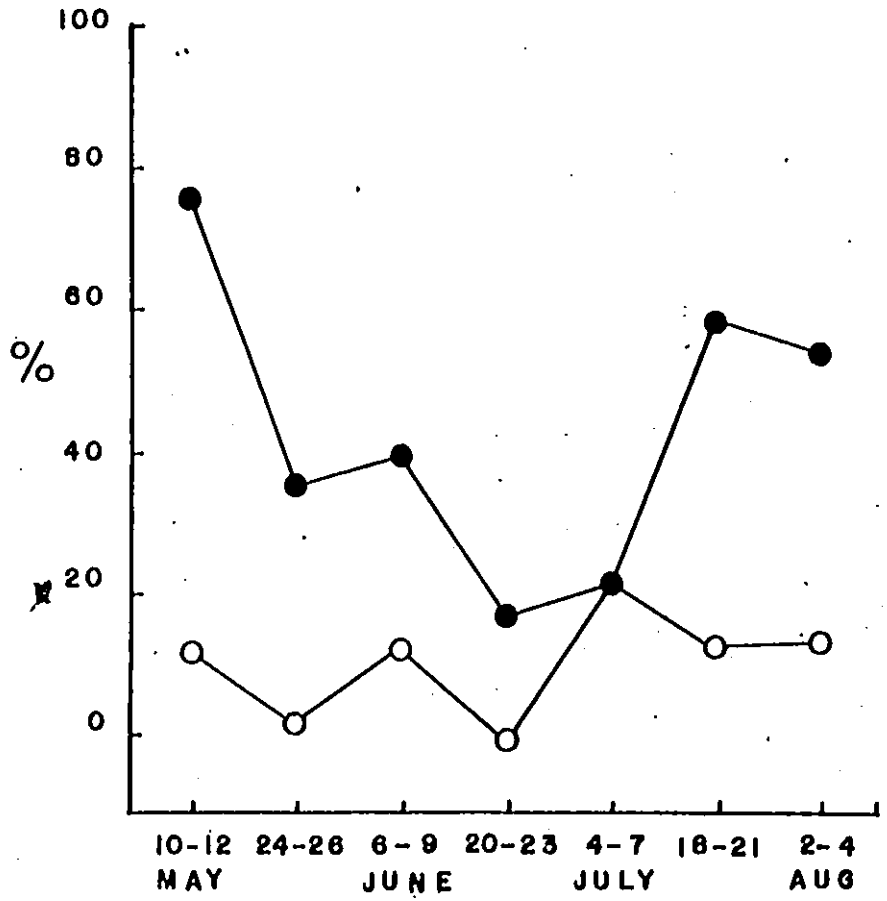

100
80
60
%
40
20
0
5-12 18-21 1-4 15-18 29-2 15-16 27-30 10-13 24-27 9-10
MAY
JUNE
JULY
AUG
SEPT

Figure 8B. Percentages of high temperature tolerant amoebae at Essex County recreational beaches in sediments and water samples (1983). Each sediment value plotted is the average of one sample taken at each of eleven beaches. Each water sample value plotted is the average of three samples at each of eleven beaches.

(●) percent high temperature tolerant amoebae in sediment samples;

(○) percent high temperature tolerant amoebae in water samples.


100
80
60
%
40
20
0
10-12 MAY
24-26
6-9 JUNE
20-23
4-7 JULY
18-21
2-4 AUG

Acanthamoeba (Data not shown), and *Naegleria* (Data not shown) than did water samples. ANOVAs of individual beaches comparing sediments and water samples showed that, at least for HTTA and *Acanthamoeba*, highly significant differences were present (Table 9). A non-significant F-value was obtained when sediments and water samples containing *Naegleria* were compared due to the low percentages of positive samples.

Site A versus Site B. In 1982, samples were taken at two distances form shore. Site A, which was only 1 meter from the shore, had a higher percentage of samples positive for HTTA and *Acanthamoeba* than did site B, which was located 5 meters from shore (Table 10). Samples containing *Naegleria* were somewhat more numerous at site B that at site A. Duplicate samples taken at site A, in 1983, showed percentages of positive HTTA and *Acanthamoeba* comparable to 1982 values, from samples taken at the same site. There was an increase in the percentage of samples containing *Naegleria* at site A, in 1983, when compared to 1982 values, at the same location.

B. J. C. Keith Power Generating Plant

During 1982, 4.49 % of the samples obtained from this plant were found to contain HTTA and *Acanthamoeba*. No samples containing *Naegleria* were isolated from this plant, however. The highest percentage of positive samples were found late in summer (Figure 9).

Water temperature showed a gradual increase over the sampling period with a decline beginning at the end of July (Figure 10). Although there was some fluctuation in air temperature, in general, a gradual increase was evident over the sampling period. Bacterial numbers were high at the beginning of the sampling period (Figure 11). They decreased by almost one order of

Table 9:

One-Way Analysis of Variance for Amoeba Populations With Respect to Type of Sample: Sediments versus Water Samples.

	Mean of Squares		F-value*
	Among Samples	Within Groups	
High Temperature Tolerant Amoebae	6429.48	51.56	124.70
Acanthamoeba	2475.99	44.75	55.33
Naegleria	1.55	15.89	0.10

*In all instances there were 1/22 degrees of freedom for the data considered. $F_{0.05}$ for 1/22 degrees of freedom equals 4.30.

Table 10:

Percentages of Positive Amoeba Samples at Different Sites on Essex County Recreational Beaches: Site A versus Site B.

	1982		1983
	Site A[a]	Site B[b]	Site A[c]
High Temperature Tolerant Amoebae	29.81	8.64	28.87
Acanthamoeba	20.67	10.45	21.39
Naegleria	0.69	0.99	2.86

[a]The percentage is based on 208 samples.
[b]The percentage is based on 220 samples.
[c]The percentage is based on 308 samples.

Figure 9. Percentages of positive amoeba samples at the J. C. Keith Power Generating Station in 1982. Each value plotted is the average of the percentages obtained at three depths at the three sites sampled.

(○) percent high temperature tolerant amoeba;
(□) percent *Acanthamoeba*.

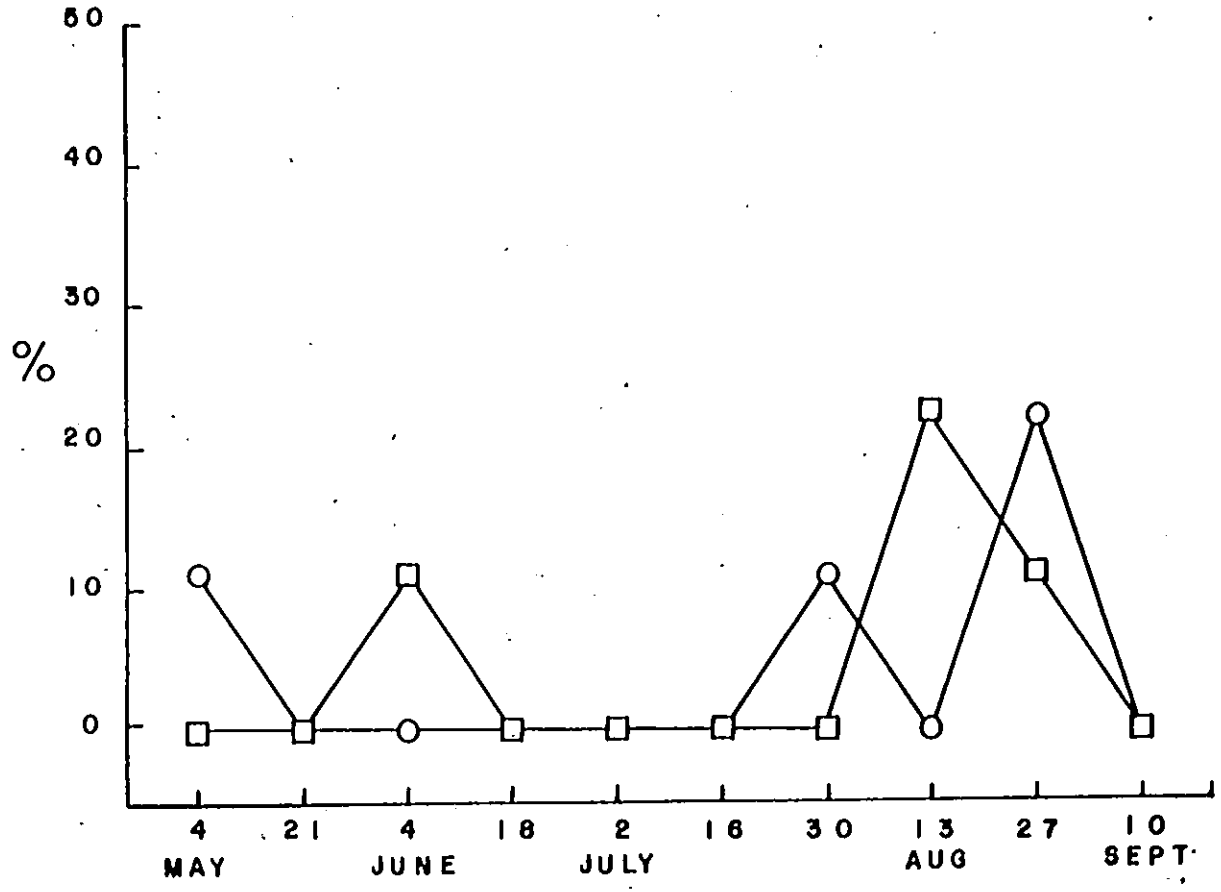

50
40
30
%
20
10
0
4
MAY
21
4
JUNE
18
2
JULY
16
30
13
AUG
27
10
SEPT

Figure 10. Temperatures measured at the J. C. Keith Power Generating Station in 1982. The air temperature value plotted is from the single measurement made at the site where heated water enters the Detroit River. The water temperature value plotted is the average of nine values obtained at three depths at each of the three sites sampled.

(□) air temperature;
(○) average water temperature.

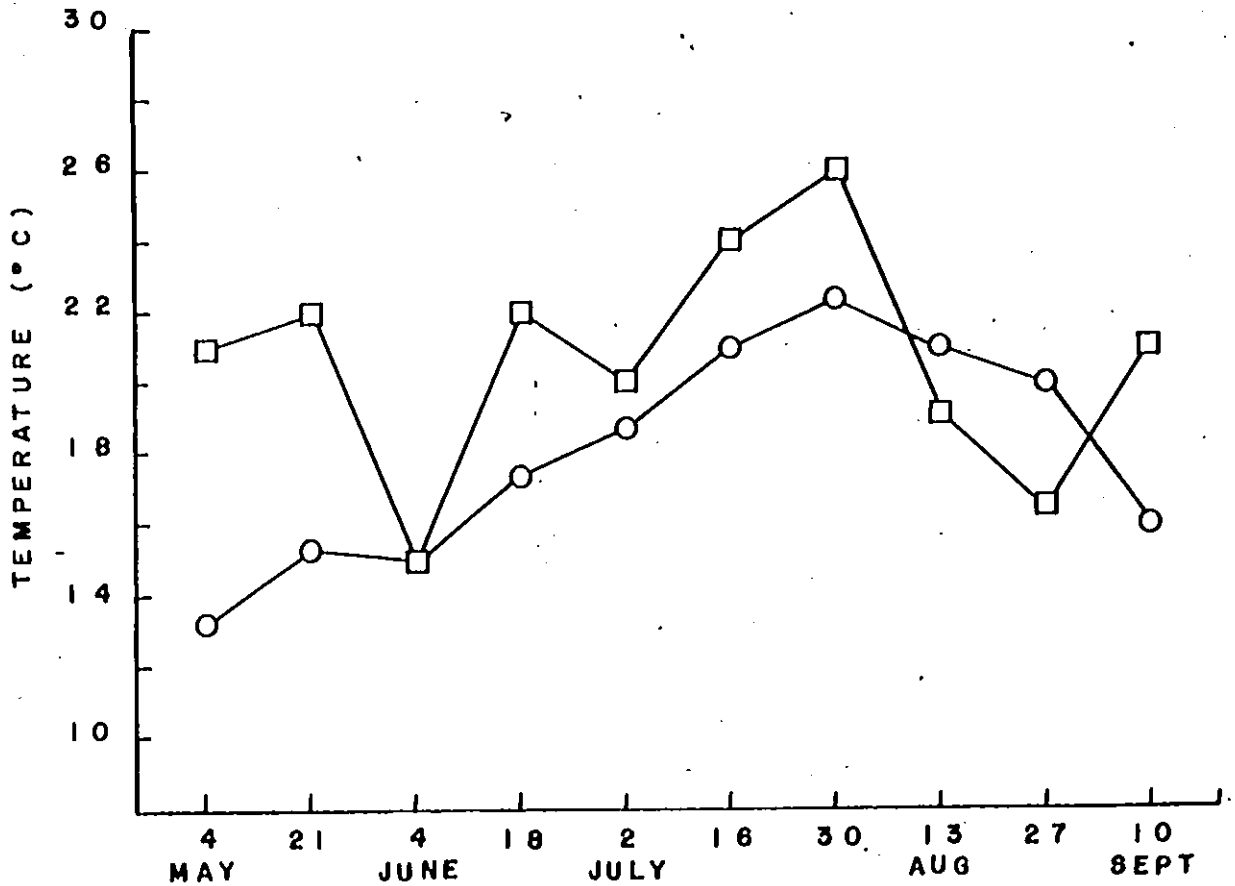
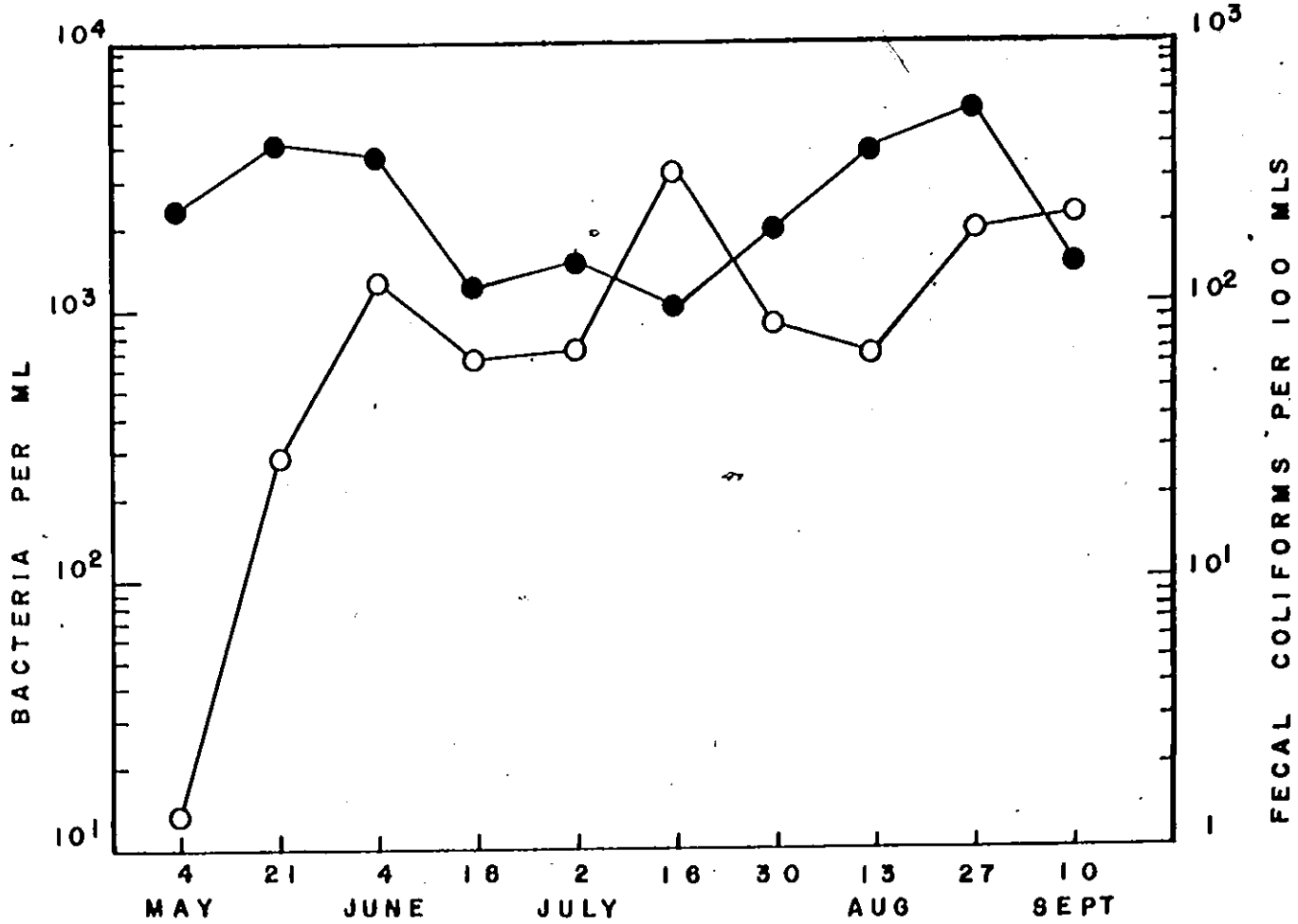

TEMPERATURE (°C)
30
26
22
18
14
10
4
MAY
21
4
JUNE
18
2
JULY
16
30
13
AUG
27
10
SEPT

Figure 11. Average bacterial and fecal coliform numbers measured at the J. C. Keith Power Generating Station in 1982. Each value plotted is the average of nine values obtained at three depths at each of the three sites sampled.

(●) bacteria per ml;
(○) fecal coliforms per 100 ml.


BACTERIA PER ML
10^4
10^3
10^2
10^1
FECAL COLIFORMS PER 100 MLS
10^3
10^2
10^1
1
4
21
4
18
2
16
30
13
27
10
MAY
JUNE
JULY
AUG
SEPT

magnitude, increased, and finally decreased again in September. Fecal coliform numbers increased by approximately two orders of magnitude within a four week period and fluctuated somewhat during the rest of the summer.

The coefficient of correlation was calculated between the parameters measured and the presence of either HTTA or *Acanthamoeba*. A statistically significant positive linear correlation was found between the presence of *Acanthamoeba* and sampling period (Table 11). Bacterial numbers were also found to be positively correlated with *Acanthamoeba* at a level of significance of 95 %. Only bacterial numbers were found to be correlated with HTTA. Due to the low variability evident in pH, no linear correlations with this parameter were calculated.

A significant negative relationship was found to exist between the distance of the sampling site from the entry point of thermally-enhanced water into the Detroit River and both bacterial and fecal coliform numbers (Data not shown).

Analysis of data obtained in a previous study at this plant (Cotter and Winner, 1981) is included along with that obtained from this study. The objective was to determine whether the depth at which the sample was taken and the distance of the sampling site from the source of heated water had any effect on the percentage of samples containing HTTA. Analysis of the earlier data showed that samples taken directly at the source of heated water had a higher percentage of positives than did samples taken from 50 and 100 meters away (Table 12). The results obtained from this study were not so clearly defined. ANOVAs showed no difference in the percentage of positive samples with increasing distance of the sampling site from the entry point of heated

Table 11:

Pearson Correlation Coefficients of Biological, Chemical and Physical Parameters Measured at the J. C. Keith Plant (1982).

Parameters	High Temperature Tolerant Amoebae	Acanthamoeba
Sampling Period	0.17703 0.1210	0.24980 0.0274
Dissolved Oxygen	- 0.10351 0.3672	0.00903 0.9375
Water Temperature	0.13580 0.2358	0.19575 0.0859
Total Bacteria	0.29398 0.0090	0.33382 0.0028
Fecal Coliforms	0.14861 0.1941	- 0.08092 0.4813

The upper number of the pair is the correlation coefficient (r). The lower number is the probability that the null hypothesis of zero correlation is correlation is correct. The correlation coefficients are based on 78 observations.

Table 12:

Effect of Distance of Sampling Site, from the Entry Point of Heated Water into the Detroit River, on the percentage of Samples Containing High Temperature Tolerant Amoebae at the J. C. Keith Power Generating Plant.

Distance (meters)	Percentage Positive Samples 1980	1981	1982
0	10.00	16.67	3.45
50	5.00	2.08	6.67
100	0.00	0.00	3.33

Percentages represent combined data at the four depths sampled.

Table 13:

Effect of Depth of Sample on the Percentage of Samples Containing High Temperature Tolerant Amoebae at the J. C. Keith Power Generating Plant.

Depth of Sample	Percentage Positive Samples 1980	1981	1982
Surface water	0.00	8.33	6.90
Mid-water	0.00	11.11	6.67
Bottom water	6.67	5.56	0.00
Sediment	13.33	0.00	ND

Percentages represent combined data at the three distances sampled.

water, at a significance level of 95 %. Table 13 shows the effects of sample depth on the percentage of samples containing HTTA. Only sediments and bottom waters were found to contain HTTA, in 1980, with sediments having the higher percentage of positives. No sediments were found to contain HTTA in 1981. Mid-water samples were found to contain a higher percentage of positives than either surface or bottom waters. In 1982, bottom waters did not contain any HTTA while surface and mid-water samples were almost equal in their percentage of positives. ANOVAs showed no difference in the percentage of positive samples with respect to depth of sample, at a level of significance of 95 %. Detailed analysis of the variation in bacterial and fecal coliform numbers with (a) distance of the sampling site from the source of heated water and (b) depth of sample, revealed a number of trends. Both bacterial and fecal coliform numbers decreased with distance of the sampling site from the source of heated water. The results are shown in Table 14. Mid-water samples showed the lowest average of both bacterial and fecal coliforms (Table 15). Surface and bottom waters had similar average concentrations of bacteria and fecal coliforms.

Samples were analysed for the presence of *Acanthamoeba*, only in 1982. As the distance of the sampling site from the source of heated water increased, from 0 to 50 and then to 100 meters, there were 3.45, 0.00 and 6.67 % positive samples, respectively. Results obtained directly at the source of entry of heated water into the Detroit River were based on 29 water samples. The results from 50 and 100 meters were each based on 30 water samples. Surface waters showed 3.45 % samples positive for *Acanthamoeba* based on 29 water samples. Mid-water and bottom water samples had 3.33 and 6.67 %

Table 14:

Effect of Distance of Sampling Site, from the Entry Point of Heated Water into the Detroit River, on Average Bacterial and Fecal Coliform Numbers at the J. C. Keith Power Generating Plant in 1982.

Distance (meters)	Total Bacteria (per ml) ± S.D.	Fecal Coliforms (per 100 ml) ± S.D.
0	3188.28 (±4440.83)	1483.21 (±1486.26)
50	1791.80 (±1649.82)	1021.38 (± 854.52)
100	1961.67 (±2865.77)	700.00 (± 691.52)

Bacterial numbers represent averages of data obtained at the three depths sampled.

Table 15:

Effect of Depth of Sample on Bacterial and Fecal Coliform Numbers at the J. C. Keith Power Generating Plant in 1982.

Depth of Sample	Total Bacteria (per ml) ± S.D.	Fecal Coliforms (per 100 ml) ± S.D.
Surface water	2323.45 (±3921.10)	1076.90 (±1046.72)
Mid-water	1969.47 (±2418.04)	799.67 (± 820.84)
Bottom water	2620.00 (±3199.82)	1082.76 (± 881.37)

Bacterial numbers represent combined data obtained at the three distances sampled.

positive results, respectively. These results were based on 30 water samples at each depth.

C. Chemical Waste Treatment Plants

The percentages of positive HTTA, *Acanthamoeba* and *Naegleria* samples present at the secondary waste treatment plant, Little River, for 1982 and 1983, are presented in Table 16. The final step in the treatment of waste material, the chlorinated effluent, is a measure of the efficiency of the treatment process in eliminating amoebae as well as bacteria and fecal coliforms. Amoebae were only found in chlorinated effluents in 1983. The amoebae present were identified as being *Acanthamoeba*. *Naegleria* were not found in any sample obtained from this plant during the two years of the study.

Chlorinated effluents obtained, in 1982, from the primary waste treatment plant, West Windsor, contained HTTA, *Acanthamoeba* and *Naegleria* (Table 17). All chlorinated effluents were found to be free of amoebae during the 1983 study.

Raw sludge samples were generally found to have the highest percentage of positive results containing HTTA and *Acanthamoeba*. The exceptions were the return activated sludge and unchlorinated effluents found at the West Windsor plant in 1982 which had only slightly higher percentages than the raw sludge samples.

D. Waste Stabilization Ponds

1. Kingsville Pond 2

Sediments obtained from this pond (Figure 12) generally had a higher percentage of samples containing HTTA and *Acanthamoeba* than did water

Table 16:

Percentage of Positive Amoeba Samples at Different Stages in the Treatment of Waste at the Little River Plant.

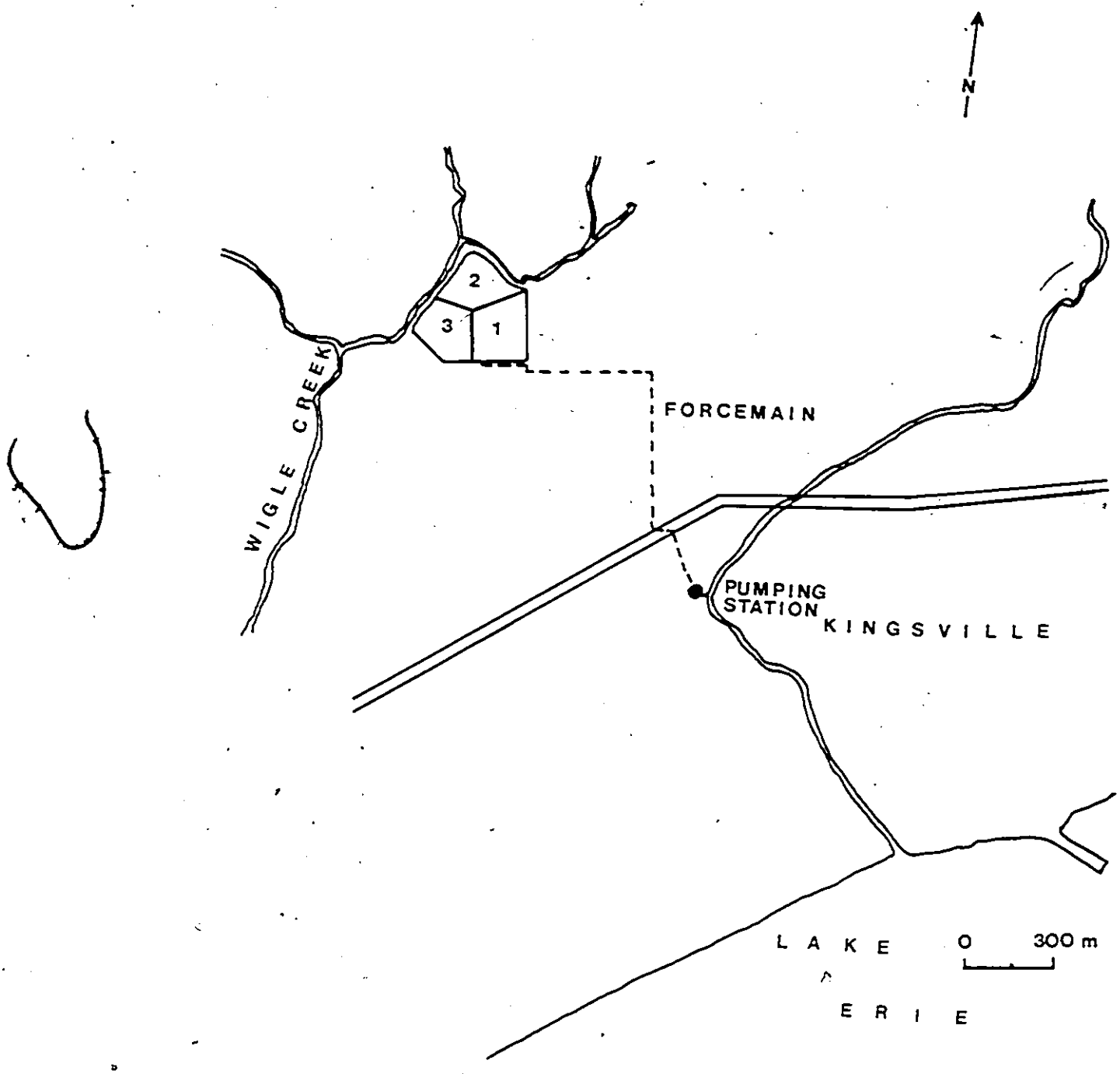
Sample	High Temperature Tolerant Amoebae		Acanthamoeba		Naegleria	
	1982	1983	1982	1983	1982	1983
Raw Sludge	77.78	85.71	77.78	71.43	0.00	0.00
Activated Sludge	50.00	ND	40.00	ND	0.00	ND
Return Activated Sludge	80.00	ND	60.00		0.00	ND
Unchlorinated Effluent	80.00	0.00	70.00	0.00	0.00	0.00
Chlorinated Effluent	0.00	0.00	0.00	14.29	0.00	0.00

Table 17:

Percentage of Positive Amoeba Samples at Different Stages in the Treatment of Waste at the West Windsor Plant.

Sample	High Temperature Tolerant Amoeba		Acanthamoeba		Naegleria	
	1982	1983	1982	1983	1982	1983
Raw Sludge	60.00	57.14	80.00	71.43	0.00	0.00
Unchlorinated Effluent	50.00	42.86	33.33	14.29	0.00	0.00
Chlorinated Effluent	30.00	0.00	20.00	0.00	30.00	0.00

Figure 12. Map showing the location of the waste stabilisation ponds sampled at Kingsville.


N
2
3
1
WIGLE CREEK
FORCEMAIN
PUMPING
STATION
KINGSVILLE
LAKE
ERIE
0
300 m

samples (Table 18). *Naegleria* were only found at Kingsville in 1982. Of all the samples collected at this site in 1982, only 44.33 % were found to contain HTTA as compared to 66.67 % in 1983. The increase in the percentage of positive HTTA was due to a large increase in the percentage of positive sediments. Increases in the percentage of samples containing *Acanthamoeba* were due to a higher percentage of positive effluents accompanied by a slight decrease in the percentage of positive water and sediment samples. An isolate of *Naegleria*, from a pond water sample was found to pathogenic in mice upon subsequent intranasal installation.

2. **Anderdon Ponds**

In 1982, at all ponds sampled (Figure 13), and at Pond 1, in 1983, a higher percentage of sediment samples were found to be positive for both HTTA and *Acanthamoeba* than water samples at the same site (Tables 19, 20). *Naegleria* were not found in either sediments or water samples in 1982. Sediments had a higher percentage of samples positive for *Naegleria*, in 1983, than did water samples. Effluents were negative for amoebae of any sort at Pond 1 in 1983.

In order to determine whether an older, established pond, such as the one at Kingsville, was more likely to contain amoebae than the recently established ponds at Anderdon, similar types of samples were examined. Since only pond sediment and water samples were obtained at the ponds during both years of study, only these samples were used in the calculations of averages presented in Table 21. Both HTTA and *Acanthamoeba* were more likely to be found at the older site than at the newer site during the two years of the study. The average percentage of samples which contained *Naegleria*,

Table 18.

Percentage of Positive High Temperature Tolerant Amoebae and *Acanthamoeba* Samples at Kingsville Pond 2.

Sample	High Temperature Tolerant Amoebae		*Acanthamoeba*	
	1982	1983	1982	1983
Pond Water	50.00	57.14	20.00	14.29
Pond Sediment	40.00	100.00	50.00	42.86
Effluent	40.00	28.57	20.00	42.86

Percentages calculated in 1982 are based on the collection of ten samples of pond water and sediment, and ten samples of effluent. The 1983 percentages are based on the collection of seven samples of pond water and sediment, and seven samples of effluent.

Table 19:

Percentage of Positive High Temperature Tolerant Amoebae and *Acanthamoeba* Samples Located in Anderdon Waste Stabilization Ponds (1982).

Pond No.	Sample	High Temperature Tolerant Amoebae	*Acanthamoeba*
1	Water	0.00	11.11
	Sediment	44.44	44.44
2	Water	0.00	0.00
	Sediment	33.33	33.33
3	Water	33.33	22.22
	Sediment	55.56	33.33

Percentages were based on the collection of nine water and nine sediment samples at each pond.

Table 20:

Percentage of Positive High Temperature Tolerant Amoebae, *Acanthamoeba* and *Naegleria* Samples at Anderdon Pond 1 (1983).

Sample	High Temperature Tolerant Amoebae	*Acanthamoeba*	*Naegleria*
Pond Water	0.00	0.00	14.29
Pond Sediment	71.43	42.86	42.86
Effluent	0.00	0.00	0.00

Percentages are based on the collection of seven samples of pond water and sediment, and seven samples of effluent.

Figure 13. Map showing the location of the waste stabilisation ponds sampled at Anderdon.

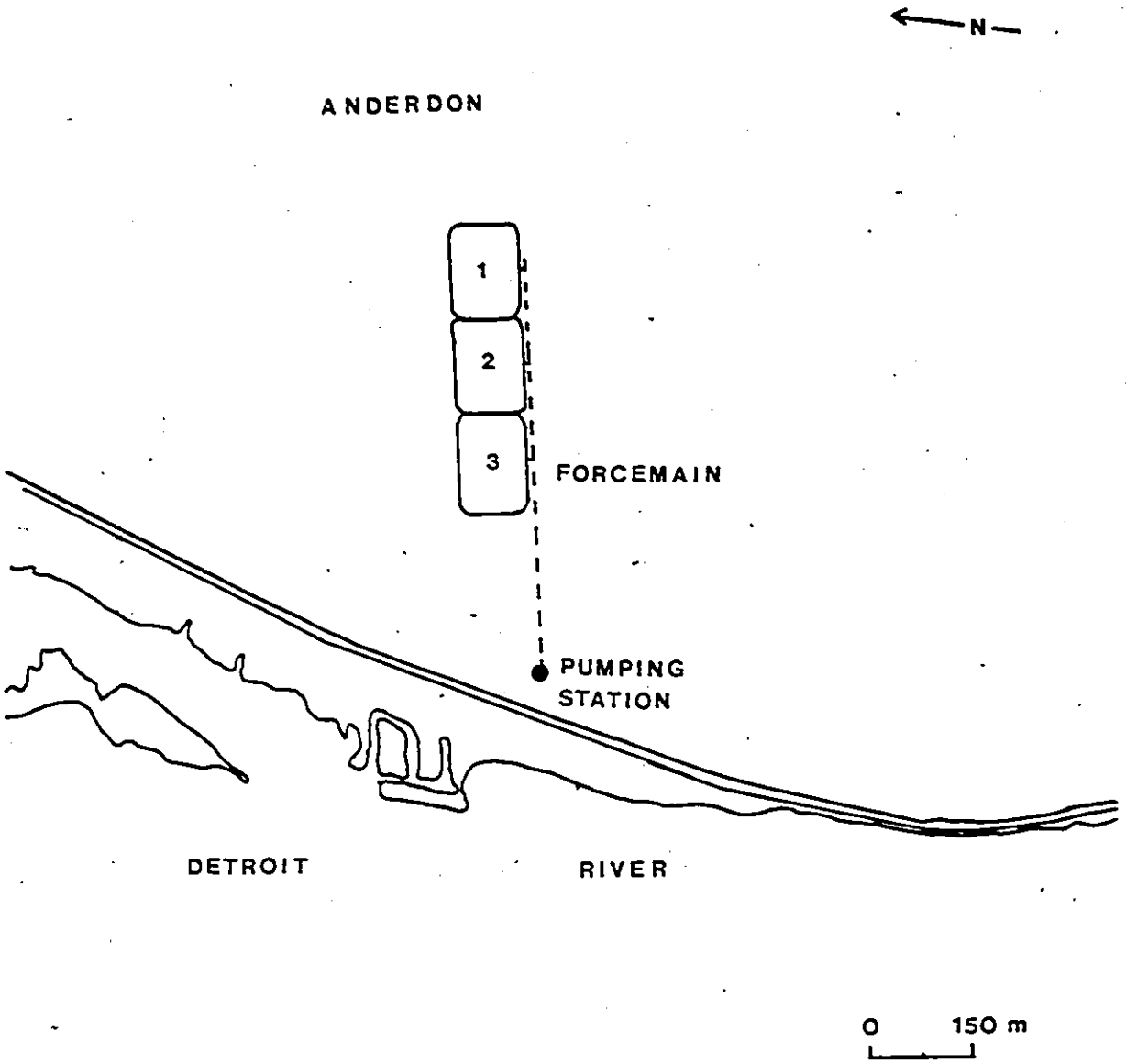

N
ANDERDON
1
2
3
FORCEMAIN
PUMPING
STATION
DETROIT
RIVER
0
150 m

Table 21

Effect of Age of Pond on the Percentage of Positive Amoeba Samples at Waste Stabilization Ponds.

Location		High Temperature Tolerant Amoebae		Acanthamoeba		Naegleria	
		1982	1983	1982	1983	1982	1983
Kingsville Pond 2 est. pre-1980		45.00	78.57	35.00	28.57	5.00	0.00
Anderdon est.							
	Pond 1	22.22	35.71	27.78	21.43	5.56	28.57
	Pond 2	16.67	ND	16.67	ND	0.00	ND
	Pond 3	44.44	ND	27.78	ND	5.56	ND
	Average	27.78		24.08		3.71	

Percentages of positive samples were calculated by averaging the totals for pond sediments and water samples. Effluents, which were sampled in 1982 and 1983, at Kingsville, and in 1983, at Anderdon, were not included in the calculations.

when calculated for all three ponds, in 1982, was lower at Anderdon than at Kingsville. In 1983, however, Pond 1 at Anderdon had 28.57 % positive *Naegleria* samples when compared to zero positive samples at Kingsville.

E. Identification of *Naegleria* Isolates

No isolates were identified as being *N. fowleri*. No isolates from Essex County beaches were found to be pathogenic in mice. On the basis of growth characteristics, mouse pathogenicity tests (Table 22) and protein patterns (Data not shown), the following beach samples are identified as *N. lovaniensis*: #2, 4, 5, 7, 8, and 10 through 13. Although no strains of *N. australiensis* were used as a control, on the basis of the other criteria tested and the fact that their protein patterns did not resemble those of either *N. fowleri* or *N. lovaniensis*, the following beach samples were identified as this species: # 1, 3, 6, 9 and 14.

At West Windsor (Table 23), sample # 1 was identified as *N. lovaniensis*, while # 2 and 3 were identified as *N. australiensis*.

Of the samples obtained at the Anderdon ponds (Table 23), # 5 through 9, only # 7 was identified as *N.australiensis* while the others were identified as *N. lovaniensis*. Sample # 4, from Kingsville, was identified as *N. australiensis*. The sample was pathogenic to mice when installed intranasally. Autopsies of the dead mice showed the presence of amoebae with the characteristics of *Naegleria*. Attempts to reintroduce the amoebae isolated from the brain into new mice failed to reproduce pathogenicity. Since the isolate had been grown with bacteria, and had therefore not lost its virulence, and since the amoebae did not show the characteristics of either *N. fowleri* or *N. lovaniensis*, it remains

Table 22:
Growth Characteristics and Mouse Pathogenicity of Naegleria Isolates from Essex County Recreational Beaches.

Beach	Site/ Date	Growth Characteristics 44.5°C with E. coli	37°C on SCGYEM	Mouse Pathogenicity (amoebae per mouse x 10^5)
1. Hillman	A Sed 06-29-82	+/-	-	0.1-0.4
2. "	A Sur 07-27-82	+	-/+	ND
3. "	B Sur 07-27-82	-	-/+	1.4-2.1
4. "	I Sed 05-10-83	+	ND	ND
5. "	II Sed 05-10-83	+	-/+	0.6
6. "	I Sur 06-20-83	-/+	ND	0.1
7. "	I Sur 07-04-83	+	-/+	2.5
8. "	II Sur 07-04-83	+	-/+	0.1
9. Pt. Pelee	B Sur 07-27-82	-	-/+	15.0
10. Mersea	II Sur 07-18-83	+/-	ND	2.5
11. "	I Sed 08-02-83	+	-/+	0.3
12. "	II Sed 08-02-83	+	-/+	2.5
13. Cedar	II Sur 07-05-83	+	-/+	0.3
14. Fox	II Sed 05-25-83	-	-	1.1

Table 22 (continued):

Two numbers presented in the mouse pathogenicity test indicate the highest and lowest amoeba concentrations injected.

Symbols.

+	= Good growth
-	= No growth
+/-	= Some growth on initial plating, poor growth on replating
-/+	= Poor growth on initial plating, no improvement on replating

Abbreviations:

A	= Site 1 meter from shore
B	= Site 5 meters from shore
I	= First beach site
II	= Second beach site
ND	= Not done
Sed	= Sediment sample
Sur	= Surface water sample
00-00-00	= Month-Day-Year

Table 23:

Growth Characteristics and Mouse Pathogenicity of Naegleria Isolates from Essex County Waste Treatment Plants and Waste Stabilization Ponds.

Location	Sample/ Date	Growth Characteristics 44.5°C with E. coli	37°C on SCGYEM	Mouse Pathogenicity (amoebae per mouse x 10^5)
1. WWP	Chlor. 05-17-82	+	-	8.6
2. "	Chlor. 06-28-82	-	ND	ND
3. "	Chlor. 07-12-82	-	-	0.3
4. Kingsville Pond	06-28-82	-/+	-/+	1.1-1.2
5. Anderdon	1. Pond 07-28-83	+/-	ND	ND
6. "	1. Sed 05-26-83	+/-	ND	3.7
7. "	1. Pond 06-21-83	-	ND	3.7
8. "	1. Sed 08-05-83	+	-/+	ND
9. "	3. Pond 07-28-82	+/-	ND	ND

Two numbers in the mouse pathogenicity test indicate the highest and lowest amoeba concentrations injected. Symbols and abbreviations in this table are as those used in Table 22.

Additional abbreviations:

WWP = West Windsor Plant
Chlor. = Chlorinated effluent
Pond = Pond water sample
Sed = Sediment sample

tentatively identified as *N. australiensis*.

DISCUSSION

Circumstantial evidence suggests that human intervention creates conditions favorable to the proliferation of pathogenic amoebae of the genera *Acanthamoeba* and *Naegleria*. Specifically, pollution, both thermal and fecal in nature, may lead to the enhancement of populations of these amoebae in public waters. It is important, therefore, to determine the relative contributions of man-made and natural sources of these kinds of pollution to populations of potentially pathogenic amoebae, especially in Essex County.

Essex County is, in effect, a peninsula. It is surrounded on the north, by Lake St. Clair on the west, by the Detroit River and on the south, by Lake Erie. The beaches found on the periphery of the county are used to a great extent during the hot, summer months.

A. Thermal Pollution

During the summer water temperatures increase naturally, resulting in the proliferation of most types of amoebae. Warmer waters are known to support the development of pathogenic *Acanthamoeba* and *Naegleria* (Stevens *et al.*, 1977; Tyndall *et al.*, 1978). Thermophilic pathogenic species appear to have a competitive advantage over other, related but non-pathogenic species during this time . The highest percentage of beach samples containing high temperature tolerant amoebae (HTTA), *Acanthamoeba* and *Naegleria* were obtained from late July to the end of August, in 1982. During this period the average water temperatures were among the highest recorded. In 1983, the highest water temperatures were recorded from the beginning of July to the beginning of August. Most HTTA, *Acanthamoeba* and *Naegleria* isolates were obtained from samples gathered during this

period. However, a higher percentage of samples positive for HTTA and *Acanthamoeba* were obtained from samples gathered from the beginning of May to the beginning of June, when water temperatures were at least ten degrees cooler. Mean water temperatures were 13.14° C (± 2.45), for the earlier period, and 23.93° C (± 1.23), for the later period. Pathogenic *N. fowleri* have been isolated from waters at temperatures as low as 16° C by Shapiro *et al.* (1982). Sykora *et al.* (1983) found that the distribution of thermophilic *Naegleria* was limited by season, at power plants in Pennsylvania. They reported the highest incidence of these amoebae in the summer and early fall. The highest percentage of samples containing HTTA and *Acanthamoba* were obtained in August, at the J. C. Keith Plant, in 1982. The highest mean water temperatures were recorded from the middle of July to the beginning of August.

Additional indications of the effect of water temperature on populations of thermophilic amoebae are evident at this plant. Samples were obtained at different distances from the discharge point of heated water into the Detroit River. It was expected that water samples taken directly at the discharge site would have a higher percentage of positives than water samples taken 50 and 100 meters away. The highest percentage of isolates containing HTTA were obtained 50 meters away from the discharge site. The highest percentage of isolates containing *Acanthamoeba* were obtained 100 meters away from the discharge site. Mean water temperatures were found to be highest at the discharge site and to decrease as the distance from the discharge site increased. Samples were also obtained at the surface of the water, the middle and the bottom. Although mean water temperatures were higher at the surface of the water than at the middle or at the bottom, the differences were

minimal. The highest percentage of samples containing HTTA were found in water obtained from the surface of the water. The percentage of mid-water samples containing HTTA was not significantly different from the percentage of positive surface water samples, 6.67 and 6.92 %, respectively. The highest percentage of samples containing *Acanthamoeba* were obtained from bottom waters. A possible explanation for the greater numbers of samples containining *Acanthamoeba* in bottom samples lies in the greater resistance of cysts of this genus to cold (Brown *et al*., 1982). The effects of thermal pollution on populations of thermophilic amoeba may also be determined by examining two recreational beaches in Essex County. The first beach, Chewitt, is located about 2 kilometers upstream from the J. C. Keith Plant, while the second beach, Holiday, is about 40 kilometers downstream. During 1982 the plant operated continuously from May to September. The mean outfall over this period was 3.4° C warmer than the inlet. A higher percentage of samples containing HTTA were obtained downstream from the plant than were found upstream. However, the reverse situation was observed for *Acanthamoeba*. In 1983, the J. C. Keith Plant was not operational during May through the beginning of August. Although the percentage of samples containing HTTA decreased at Chewitt in 1983, when compared to 1982 (-3.16), the decrease was comparable to the overall changes seen for the other beaches sampled. These beaches were located both far upstream and downstream from the J. C. Keith Plant. Holiday beach, located downstream from this plant, showed a decrease of 194.86 % in 1983. This suggests a real, if limited role for thermal pollution in affecting the populations of thermophilic amoebae at Holiday beach. As in 1982, a higher percentage of samples containing *Acanthamoeba* were obtained at the beach upstream from

the power plant. Therefore, although warmer waters generally contain a higher percentage of these thermophilic amoebae, exceptions are possible. Other factors determining the presence or absence of these amoebae must be considered.

B. Fecal Pollution

Even though water temperatures may be favorable for the growth of thermophilic, potentially pathogenic amoebae, if an adequate food source is not available, growth will not occur. Mean bacterial numbers were not found to be correlated in a positive linear fashion with the percentage of beach samples containing either HTTA or *Acanthamoeba*. However, a weakness in the methodology employed was the absence of any measurement of bacterial and coliform numbers present in sediments. The majority of positive results were obtained from these samples. The reason for not measuring these parameters will be discussed later. Such measurements may have been of limited use. Pathogenic free–living amoebae ingest yeast, bacteria and organic debris. The relative contributions of each food source to their diet has not been determined. In addition, not all species of bacteria are suitable food sources for these amoebae (Singh, 1975). Competition for bacteria by other species of amoebae must also reduce the number of bacteria available for these thermophilic species. Danso and Alexander (1975) and Danso *et al.* (1975) report that although there was an inverse relationship between the number of active amoebae and bacteria, both *Acanthamoeba* and *Naegleria* were unable to totally eliminate the food source. Therefore, there must exist some minimum number of bacteria required for the coexistence of both bacteria and amoebae. In addition, a limit on maximum bacterial density must also exist. This limit is probably determined by factors

including competition for available nutrients and predation. Similar limitations must exist for thermophilic free-living amoebae. At the J. C. Keith Plant, where bacterial numbers were determined for each sample taken, a significant ($P > 95$ %) positive linear correlation was found, between bacterial numbers and the percentage of samples containing HTTA and *Acanthamoeba*.

Fecal coliforms were measured as a subset of the bacterial population. These bacteria are part of the normal flora of the human digestive system and their numbers are an indication of the relative pollution from human sewage. Recreational beaches containing an excess of 100 fecal coliforms per ml are considered unsafe for swimming and other water sports. The proliferation of *N. fowleri* on *E. coli* and the isolation of pathogenic strains from sewage (Singh and Das, 1972) suggested to De Jonckheere (1978) that high densities of *E. coli* were required for the presence of *N. fowleri* in surface waters. However, De Jonckheere found that the density of fecal coliforms increased with distance from the source of heated water. This was the reverse of the situation for *N. fowleri*, which decreased with distance from the source of heated water. Apparently, *E. coli* was not able to survive at the higher temperatures. At the J. C. Keith Plant both the total bacterial and fecal coliform numbers decreased with distance from the source of heated water. This would suggest that water temperatures were not so high as to result in death of the bacteria (temperatures optimal for the growth of the bacteria had not been reached). Both total bacterial and fecal coliform numbers were almost as high in bottom waters at the plant as in surface waters. However, no HTTA were found in bottom waters. If, as mentioned earlier, the *Acanthamoeba* present were in the form of cysts at

this depth, the high bacterial and fecal coliform numbers would suggest few amoebae actively feeding.

The effects of fecal pollution on populations of potentially pathogenic amoebae were observed more directly at the waste treatment plants and waste stabilization ponds sampled. Raw sludge samples generally had the highest percentage of samples containing both HTTA and *Acanthamoeba*. *Naegleria* were not isolated, presumably due to overgrowth, either in the sludge or upon culture in the laboratory. Such overgrowth is not unknown when mixed cultures are present during incubation. After the raw sludge is allowed to settle, aerobic bacteria are added, at secondary waste treatment plants. The resulting mixture, called the activated sludge, is aerated. This treatment reduces the total bacterial load as well as the sewage content. A portion of this mixture, called the return activated sludge, is returned to a holding tank for future use. Both HTTA and *Acanthamoeba* were found in this mixture. At primary waste treatment plants raw sludge is allowed to settle and larger pieces of waste are screened out. The supernatant goes directly to the next step of treatment as unchlorinated effluent. Again, both HTTA and *Acanthamoeba* were found in a great percentage of the samples obtained. Chlorination, the final step, was found to be more efficient in eliminating HTTA than *Acanthamoeba*, at the secondary waste treatment plant. These results are indicative of the resistance of cysts of *Acanthamoeba* to chlorination, as discussed in the **INTRODUCTION**. If *Naegleria* had been present in unchlorinated effluents, they were most likely again overgrown by other HTTA or *Acanthamoeba*. Cysts of *Naegleria*, if present, did not appear to survive chlorination. This is in agreement with what is known about their resistance to chlorine (De Jonckheere and Van Voorde, 1976).

At the primary waste treatment plant chlorination did not eliminate either HTTA, *Acanthamoeba* or *Naegleria*, during 1982. Chlorination at this plant was known to be speradic and chlorine levels were not always maintained sufficiently high to eliminate either potentially pathogenic amoebae or bacteria. In the majority of instances in which amoebae were found in chlorinated effluent samples, high total bacterial numbers and/or high fecal coliform numbers were also measured. Chlorinated effluent samples which contained *Naegleria* did not contain *Acanthamoeba*. It is possible that in mixed cultures of these two genera, with very low levels of chlorine, *Naegleria* were able to grow better than *Acanthamoeba*. As a result, there were more samples containing *Naegleria* than *Acanthamoeba*. Waste treatment plants served as reservoirs for HTTA, *Acanthamoeba* and, on at least three occasions, *Naegleria*. The primary waste treatment plant was more likely to act as a reservoir than the secondary waste treatment plant.

Waste stabilization ponds at Kingsville and Anderdon acted both as reservoirs and dissemination points for potentially pathogenic amoebae. HTTA, *Acanthamoeba* and *Naegleria* may overwinter as cysts in the sediments, which do not freeze during the winter. Rapid freezing is known to be more detrimental to cysts of pathogenic rather than non-pathogenic *Naegleria*. This may account for the absence of pathogenic, although not of non-pathogenic *Naegleria*. Although HTTA, *Acanthamoeba* and *Naegleria* could be found in pond water or effluents, the highest percentage of positive results were obtained from sediments, in all but one instance. Most effluent samples were found to contain all three types of amoebae. From Anderdon the effluents empty directly into the Detroit River.

The effluents from Kingsville drain into Wigle Creek, which then empties into Lake Erie. In this fashion these potentially pathogenic amoebae may be cycled through the southern portion of the Detroit River and Lake Erie. Wind dispersal and agricultural runoff probably accounts for the presence of HTTA and *Acanthamoeba* in Lake St. Clair and the northern portion of the Detroit River. Cysts of *Naegleria* are less resistant to drying than those of *Acanthamoeba* (Chang, 1978). Pathogenic *Naegleria* are especially susceptible to drying. This may explain the absence of *Naegleria* from these latter areas.

C. Chemical Parameters: pH and Dissolved Oxygen

Measurements of pH were taken at the beaches and J. C. Keith Power Plant during 1982. pH levels were found to remain constant at 8.5 during the entire sampling period, from the beginning of May to the beginning of September. Sykora *et al.* (1983) found that pathogenic trophozoite numbers decreased after cysts were cultured at a pH in excess of 8.7. No trophozoites grew at a pH of 10. The absence of *Naegleria*, at the power plant, and of pathogenic *Naegleria*, at the beaches, may be due to the alkaline nature of the water (Brown *et al.*, 1983).

Naegleria is known to have an aerobic metabolism. Pathogenic strains consume and probably require less oxygen than non-pathogenic strains (John, 1982). Dissolved oxygen content decreases with increases in water temperature. The appearance of pathogenic *Naegleria* under conditions of lower oxygen content is probably related to their lower oxygen requirements. *Naegleria* were isolated from recreational beaches, in Essex County, with dissolved oxygen levels from 8 to 14 ppm (Mean dissolved oxygen content = 9.94 ± 1.98). Dissolved oxygen levels varied from a minimum of 5.2 to a maximum

of 14 ppm during the two years they were measured at Essex County beaches. At the J. C. Keith plant dissolved oxygen levels varied from a minimum of 5.2 to a maximum of 10.3 ppm. Neither HTTA nor *Acanthamoeba* isolates showed any correlation with dissolved oxygen content, at either the beaches or the power plant.

Parameters such as dissolved oxygen content and pH cannot be used to predict whether HTTA, *Acanthamoeba* or *Naegleria* will be present in a body of water. However, they may suggest reasons as to why the amobae may be present or absent.

D. Methodology

1. Sample Volume and Type

No pathogenic *N. fowleri* were detected at any location sampled in Essex County. Both *N. lovaniensis* and *N. australiensis* were isolated at beaches on Lake Erie. An earlier survey sponsored by the Department of Health and Welfare in 1979 (Seyfried *et al.*) reported the presence of pathogenic *N. fowleri* at Holiday Beach. Subsequent studies in 1980, at the same beach, by these workers, failed to find either pathogenic or non-pathogenic *Naegleria*. Low numbers of amoebae in 1980 seem to be the best explanation for these findings. Our own results may therefore simply reflect low concentrations of pathogenic *Naegleria* at these beaches.

De Jonckheere (1978b) reports that water samples of 1 to 10 ml are more likely to contain pathogenic *N. fowleri* than larger samples of up to 250 ml. The selection of a sample size of 100 ml used in this study was based on earlier work in our laboratory, at power generating plants. Lower concentrations of amoebae were present at the plants than were subsequently found in recreational beaches. Although De Jonckheere found that the

percentage of non-pathogenic *Naegleria* increased, in larger sample volumes, the percentage of pathogenic *Naegleria* strains isolated, decreased. Sampling organic solids or sediments produced the same results as increasing the volume of water samples. Sykora *et al.* (1983) found that, in locations which contained thermophilic, non-pathogenic *Naegleria*, no pathogenic strains were to be found. The pathogenic strain was the only *Naegleria* isolate obtained from a particular locality, on several occasions. It appears that when pathogenic and non-pathogenic *Naegleria* are found together, the non-pathogens will overgrow the pathogens. Therefore, although the presence of non-pathogenic *Naegleria* in a locality indicates that conditions may be favorable for the growth on pathogenic *Naegleria*, the non-pathogens may prevent the pathogens from proliferating. Only when conditions are especially suitable for the growth of pathogenic *Naegleria*, i.e. very high water temperatures, may we be able to isolate pathogenic *Naegleria*. In general, water temperatures at both beaches and the electric power plant were not high enough or elevated for a sufficient period to allow pathogenic *Naegleria* to overgrow non-pathogenic strains.

Neither total bacterial nor fecal coliform numbers were determined for sediment samples during the two years of the study. Previous work done in our laboratory, at three power generating plants in Southern Ontario found that sediments generally contained high numbers of both types of bacteria. However, there was a very high variability in numbers which required dilutions of the test material from 10^1 to 10^6. Such work expended a great deal of both time and material. The results obtained did not warrant the additional work. On the basis of these observations, it was decided not to

measure these two biological parameters for sediment samples. However, the results obtained in 1982, at the J. C. Keith Plant, suggests that their usefulness must be reevaluated. Sludge samples at the waste treatment plants were also not monitored for total bacterial and fecal coliform numbers, for the above reasons.

2. Isolation Procedure

A solution to the problem of overgrowth by non-pathogens was proposed by De Jonckheere (1978b). Centrifugation of larger water volumes, followed by spreading of the pellet over non-nutrient-agar, seeded with bacteria, resulted in the formation of individual plaques. The amoebae in the plaques could then be tested for the presence of pathogenic amoebae. This procedure could not be used because desiccation of the agar occurred during prolonged incubation. Instead, agar plates were flooded with distilled water containing *E. coli* B/r. As a result, non-pathogens probably overgrew the pathogens due to the faster growth of the former.

An initial incubation temperature of 41° C was used to allow the growth of potentially pathogenic *Acanthamoeba*. As mentioned earlier (INTRODUCTION), pathogenic *Acanthamoebae* are able to tolerate temperatures as high as 43° C. De Jonckheere (1979c) reports that such preincubation at lower temperatures is detrimental to the isolation of pathogenic *Naegleria*.

3. Mouse Pathogenicity

Tests for mouse pathogenicity of HTTA, and *Acanthamoeba* were not performed due to the large numbers of samples obtained. It would have taken a total of 3.07 years (6 samples at a time with a 21 day incubation period) and 1595 mice (3-4 weeks old, 5 mice per sample) to perform the intranasal

installation tests, on the beach samples alone. The time period and animal requirements would have doubled if intracerebral injections had also been performed. Autopsies of test animals, both dead and surviving would also have been required.

This study revealed that pathogenic *Naegleria* are not present at recreational beaches in Essex County. Thermal effluents from the J. C. Keith Power Generating Plant have a limited role in contaminating large bodies of generally colder water such as the Detroit River with potentially pathogenic amoebae. Smaller systems such as waste stabilization ponds may remain contaminated and serve as reservoirs for future contamination under more favorable environmental conditions. Primary waste treatment plants with low chlorine levels may also serve as reservoirs for these amoebae. In general, secondary waste treatment plants are able to eliminate most types of contaminating amoebae. However, the resistant nature of *Acanthamoeba* cysts indicates that superchlorination is required to eliminate this species from effluents.

As the number of individuals using the Great Lakes increases, their demands for freshwater, i.e., for electrical power production, recreation and as a dumping ground for sewage, will also increase. It is not inconceivable, that at this time, conditions may be favorable for the growth of these pathogenic, free–living amoebae. Therefore, a permanent system of monitoring the sizes and locations of populations of these amoebae should be developed.

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APPENDIX

Table 24:
Standard Deviations for Percentages of Positive Amoeba Samples at Essex County Recreational Beaches During 1982.

Date	High Temperature Tolerant Amoeba	Acanthamoeba	Naegleria
May 5 - May 12	16.49	22.29	0.00
May 18 - May 21	9.73	12.87	0.00
June 1 - June 4	9.73	9.73	0.00
June 15 - June 18	18.46	13.06	0.00
June 29 - July 2	16.71	12.23	7.22
July 13 - July 16	9.73	7.22	0.00
July 27 - July 30	24.91	27.09	16.28
Aug. 10 - Aug. 13	18.46	16.71	0.00
Aug. 24 - Aug. 27	16.71	29.11	0.00
Sept. 9 - Sept. 10	0.00	0.00	0.00

Percentages in September are based on a total of eight samples taken at two beaches. All other percentages are based on samples taken at twelve beaches.

Table 25:
Standard Deviations for Percentages of Positive Amoebae Samples at Essex County Recreational Beaches During 1983.

Date	High Temperature Tolerant Amoebae	Acanthamoeba	Naegleria
May 10 - May 12	21.85	21.85	7.54
May 24 - May 26	28.00	17.19	7.54
June 6 - June 9	17.52	19.66	0.00
June 20 - June 23	12.61	11.68	7.54
July 4 - July 7	20.71	23.11	16.11
July 18 - July 21	30.34	23.60	7.54
Aug. 2 - Aug. 4	16.85	21.85	10.11

Table 26:
Standard Deviations for Temperatures Measured at Essex County Recreational Beaches in 1982.

Date	Air Temperature (°C)	Water Temperature (°C)
May 5 - May 12	4.05	3.02
May 18 - May 21	4.50	1.14
June 1 - June 4	3.74	1.79
June 15 - June 18	2.38	1.06
June 29 - July 2	2.17	1.41
July 13 - July 16	3.77	1.64
July 27 - July 30	2.08	0.40
Aug. 10 - Aug. 13	2.29	0.95
Aug. 24 - Aug. 27	3.42	0.89
Sept. 9 - Sept. 10	3.00	4.00

The September data is based on temperatures taken at only the two beaches sampled. The rest of the data is based on temperatures taken at twelve beaches.

Table 27:
Standard Deviations for Temperatures Measured at Essex County Recreational Beaches in 1983.

Date	Air Temperature (°C)	Water Temperature (°C)
May 10 - May 12	2.90	0.93
May 24 - May 26	2.94	1.52
June 6 - June 9	3.01	1.02
June 20 - June 23	3.16	2.39
July 4 - July 7	1.61	1.52
July 18 - July 21	2.04	2.56
Aug. 2 - Aug. 4	2.23	1.23

Table 28:
Standard Deviations for Average Bacterial and Fecal Coliform Numbers Measured at Essex County Recreational Beaches in 1982.

Date	Total Bacteria (per ml)	Fecal Coliforms (per 100 ml)
May 5 - May 12	998.44	8.06
May 18 - May 21	57303.00	36.08
June 1 - June 4	5473.66	121.30
June 15 - June 18	10997.87	91.23
June 29 - July 2	2138.39	54.63
July 13 - July 16	1877.38	29.68
July 27 - July 30	1012.03	409.67
Aug. 10 - Aug. 13	19412.68	33.37
Aug. 24 - Aug. 27	1898.54	62.67
Sept. 9 - Sept. 10	4652.76	79.90

The September data is based on samples taken from two beaches. The rest of the data is based on samples at twelve beaches.

Table 29:
Standard Deviations for Average Bacterial and Fecal Coliform Numbers Measured at Essex County Recreational Beaches in 1983.

Date	Total Bacteria (per ml)	Fecal Coliforms (per 100 ml)
May 10 - May 12	4166.43	112.25
May 24 - May 26	1278.46	62.87
June 6 - June 9	3761.04	123.14
June 20 - June 23	3820.38	71.90
July 4 - July 7	10818.02	54.04
July 18 - July 21	84535.02	82.11
Aug. 2 - Aug. 4	3431.51	43.65

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